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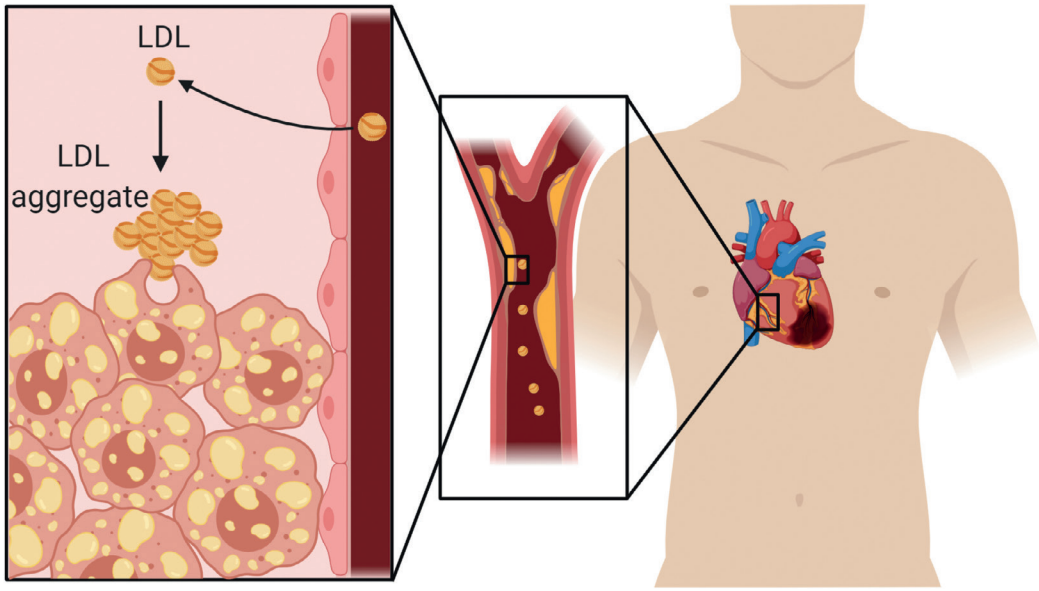
MAIJA RUUTH

LDL AGGREGATION SUSCEPTIBILITY AS A NOVEL RISK FACTOR FOR ATHEROSCLEROSIS



WIHURI RESEARCH INSTITUTE AND
MEDICUM
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN CLINICAL RESEARCH
UNIVERSITY OF HELSINKI

LDL AGGREGATION SUSCEPTIBILITY AS A NOVEL RISK FACTOR FOR ATHEROSCLEROSIS

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DOCTORAL DISSERTATION

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There is only one duty ~ beauty

Only one reality ~ dream

Only one power ~ love

Armi Ratia

To all with whom I have shared beauty, dreams, and love

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Maija Ruuth*, Laura G M Janssen*, Lauri Äikäs, Feven Tigistu-Sahle, Kimberly J Nahon, Olli Ritvos, Hanna Ruhanen, Reijo Käkelä, Mariëtte R Boon, Katariina Öörni[§], Patrick C N Rensen[§]; LDL aggregation susceptibility is higher in healthy South Asian compared with white Caucasian men, *Journal of Clinical Lipidology,* Volume 13, Issue 6, Pages 910-919, 30 September 2019. *,[§]Equal contribution.

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AUTHOR´S CONTRIBUTIONS

I The author participated in the study design, conducted all the experiments and analyses with the exception of aggregation susceptibility measurements of Health 2000, Sysdiet, and the SOAT$^{-/-}$ mouse samples, and lipidomic analyses of LDL from Health 2000 and Corogene LDL samples and samples from mice injected with large empty vesicles. Animals for SOAT$^{-/-}$ and those treated with large empty vesicles were housed in Prof. Rudel's lab and Prof. Boréns lab from where samples were collected. Animals used in the myriocin experiment were housed in Kuopio and myriocin was injected by Dr. Huusko. The author wrote the first draft of the manuscript, which was then commented on by the other authors.

II The author participated in the study design, conducted all the experiments and analyses with the exception of collecting study participant baseline characteristics and performing the lipidomic mass spectrometry measurement. The author wrote the first draft of the manuscript, which was then commented on by the other authors.

III The author participated in the study design, conducted all the experiments and analyses with the exception of collecting study participant baseline characteristics and performing the lipidomic mass spectrometry measurement. The author wrote the first draft of the manuscript, which was then commented on by the other authors.

ABBREVATIONS

ASCVD	atherosclerotic cardiovascular disease
apo	apolipoprotein
BMI	body mass index
CAD	coronary artery disease
CD	circular dichroism
CE	cholesterol ester
Cer	ceramide
CETP	cholesteryl ester transfer protein
CVD	cardiovascular disease
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FFA	free fatty acid
HDL	high density lipoprotein
hsCRP	high sensitivity C-reactive protein
IL	interleukin
LDL	low density lipoprotein
LDL-C	low density lipoprotein cholesterol
LDLR	LDL-receptor
Lp(a)	lipoprotein (a)
LPC	lysophosphatidylcholine

LPL	lipoprotein lipase
mAb	monoclonal antibody
MI	myocardial infarction
MMP	matrix metalloprotease
MUFA	monounsaturated fatty acid
oxLDL	oxidized LDL
PAD	peripheral artery disease
PC	phosphatidylcholine
PCSK9	proprotein convertase subtilisin/kexin type 9
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PL	phospholipid
PLA_2	phospholipase A_2
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
SM	sphingomyelin
SMase	sphingomyelinase
SOAT	sterol-O-acyltransferase
TAG	triacylglycerol
TG	triglyceride
UC	unesterified cholesterol
VLDL	very low density lipoprotein

ABSTRACT

Atherosclerotic cardiovascular disease (ASCVD) is the leading worldwide cause of mortality and morbidity, being both an economic burden for healthcare systems and a source of great individual suffering. The low density lipoprotein cholesterol (LDL-C) concentration in plasma is a causal and modifiable risk factor for ASCVD.

Atherogenesis is initiated and then driven by retention, modification, and aggregation of low density lipoprotein (LDL) particles in the arterial intima. Experimental work by our group and others has demonstrated that aggregated LDL particles can induce lipid accumulation in macrophages and this can also activate intimal cells and thereby induce inflammation in the arterial wall. Here, it was hypothesized that it is not only the plasma concentration of LDL particles that influences atherogenesis, but also their characteristics. This thesis aims at testing whether subjects with aggregation-prone LDL have an increased risk for ASCVD and/or ASCVD death. For this purpose, a method to measure LDL aggregation susceptibility was developed, and it was further studied if LDL aggregation susceptibility is modifiable. In addition, it is studied here if LDL aggregates cause a heightened inflammatory response in cells present in the artery wall.

The first publication was a hypothesis-generating study, where it was discovered that LDL particles from ASCVD patients are more prone to aggregate in comparison to those from healthy individuals, and importantly, that aggregation-prone LDL predicted future ASCVD death in a group of patients with established ASCVD. It was found that the aggregation-prone LDL particles are rich in sphingolipids but have less phosphatidylcholines than their aggregation-resistant LDL counterparts. Three interventions in animal models aimed at altering the LDL composition, were observed not only to lower the susceptibility LDL particles to aggregate but also to slow the development of atherosclerosis. Similar compositional changes induced in humans by proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition or adoption of a healthy Nordic diet also lowered the LDL aggregation susceptibility.

In the second publication it was demonstrated that genetic background, here ethnicity, influenced LDL aggregation susceptibility. LDL particles from South Asians were more

prone to aggregate compared to those from white Caucasians, a finding that may partly explain why South Asians are at higher risk for ASCVD.

The third study was a dietary intervention to investigate if different macronutrients could alter LDL particles aggregation susceptibility. Saturated fats were found to increase LDL aggregation, while unsaturated fats or simple sugars had no effect.

In addition, the consumption of plant stanol esters, that are known to reduce the LDL-C concentration, was found to decrease the LDL aggregation susceptibility.

This thesis propose that the aggregation susceptibility of LDL particles is a novel modifiable risk factor of ASCVD; its assessment may add predictive power to the conventional ASCVD risk estimation. The evaluation of this biomarker may facilitate the identification of those patients who would benefit most from aggressive LDL-C-lowering therapies.

TIIVISTELMÄ

Ateroskleroosi eli valtimonkovettumatauti on maailman yleisin kuolinsyy ja se kehittyy vuosien kuluessa lapsuudesta lähtien. Plasman LDL-hiukkasten kolesterolipitoisuus on merkittävä riskitekijä taudin kehittymiselle, mikä on näytetty geneettisissä, epidemiologisissa ja kliinisissä tutkimuksissa.

LDL-hiukkasten pitoisuus valtimon seinämän sisäkerroksessa, intimassa, on sama kuin plasmassa. LDL-hiukkasten kertyminen valtimon seinämään alkaa plasman LDL kolesteroli pitoisuuden ollessa suurempi kuin fysiologinen LDL kolesteroli pitoisuus (1-1,5 mmol/l). Intimassa LDL-hiukkaset takertuvat solunulkoiseen tukiverkkoon ja ovat alttiita entsymaattisille ja hapettaville muutoksille. Muuntuneet LDL-hiukkaset ovat alttiita aggregoitumaan eli takertumaan toisiinsa, ja aggregoituneet LDL-hiukkaset puolestaan tarttuvat entistä tiukemmin soluväliaineeseen. LDL-hiukkasten kerääntyminen intimaan houkuttelee paikalle makrofageja, jotka fagosytosoivat erityisesti aggregoituneita hiukkasia, mikä johtaa vaahtosolujen muodostumiseen ja paikalliseen tulehdukseen. Valtimoplakkien tiedetään sisältävän aggregoituneita LDL-hiukkasia ja eläinkokeissa on näytetty, että plasman LDL aggregoituu valtimon seinämässä vain kahden tunnin kuluessa siitä, kun eläimeen injektoidaan LDL-hiukkasia ja plasman LDL-kolesterolipitoisuus kohoaa.

Tässä väitöskirjatyössä kehitin menetelmän mitata LDL-hiukkasten aggregoitumisherkkyyttä verinäytteistä. Osoitin, että LDL-hiukkasten herkkyys aggregoitua vaihtelee ihmisten välillä ja johtuu eroista LDL-hiukkasten pinnan rasvojen suhteista, fosfatidyylikoliinien ja sfingomyeliinien suhteellisesta osuudesta. Sfingomyeliiniä paljon sisältävät LDL-hiukkaset aggregoituvat herkemmin, ja fosfatidyylikoliinia paljon sisältävät LDL-hiukkaset hitaammin. Osoitin, että LDL-hiukkaset aggregoituvat herkemmin sepelvaltimotautipotilailla kuin terveillä verrokeilla ja mikä tärkeintä, LDL- hiukkasten aggregoitumisherkkyys ennusti tulevaa sydänkuolemaa sepelvaltimotautia sairastavilla potilailla. LDL-hiukkasten aggregoitumisherkkyys osoittautui itsenäiseksi riskitekijäksi, eikä korreloinut esimerkiksi plasman LDL-kolesterolipitoisuuden tai iän kanssa, eikä eronnut miesten ja naisten välillä. Lisäksi osoitin, että LDL-hiukkasten herkkyys aggregoitua riippuu mahdollisesti ainakin osittain geneettisistä tekijöistä, sillä eteläaasialaisten LDL aggregoitui herkemmin kuin

länsimaalaisten.

Kliinisesti on tärkeää tietää voiko LDL-hiukkasten aggregoitumisherkkyyteen vaikuttaa elintavoilla tai lääkityksellä. Näytin tässä työssä, että ravinnon rasvoilla on merkitystä, sillä tyydyttyneet rasvat huononsivat LDL-hiukkasten laatua ja lisäsivät LDL-hiukkasten aggregoitumisherkkyyttä.

Pohjoismainen terveellinen ruokavalio, erityisesti siihen kuuluvat kasviöljyt paransivat LDL-hiukkasten laatua ja vähensivät aggregoitumista. PCSK9 (proprotein convertase subtilisin/kexin 9) estäjä, plasman kolesterolipitoisuutta laskeva monoklonaalinen vasta-aine lääke vähensi LDL-hiukkasten aggregoitumista parantamalla LDL-hiukkasten laatua.

Lisäksi havaittiin, että kasviöljypohjaisen kasvistanolia sisältävän levitteen käyttö puolestaan paransi LDL-hiukkasten laatua ja vähensi LDL-hiukkasten aggregoitumisherkkyyttä.

Tämä väitöskirjatyö esittelee LDL-hiukkasten aggregoitumisherkkyyden uutena muokattavissa olevana riskitekijänä ateroskleroottisille valtimosairauksille. LDL-hiukkasten aggregoitumisherkkyys voi tulevaisuudessa auttaa tunnistamaan potilaat, jotka hyötyisivät eniten aggressivisesta LDL kolesterolia alentavasta lääkityksestä.

1 INTRODUCTION

Cardiovascular diseases (CVDs) are the most common global cause of death and this is also the case in Finland (1). CVDs caused an estimated 17.6 million deaths in 2016, which represents 32% of all global deaths, and 70% of these deaths are due to events related with atherosclerotic disease such as heart attack and stroke. CVDs can be prevented to some extent by managing behavioural risk factors; of these, the most important detrimental factors are unhealthy diet, physical inactivity, smoking and excess use of alcohol (2). These behavioural risk factors are associated with physical outcomes such as increased blood lipids, elevated blood pressure, high blood glucose, and overweight/obesity. These risk factors can be measured in primary care to evaluate the need for lifestyle changes and/or medication.

The low density lipoprotein cholesterol (LDL-C) concentration in plasma is a well-established independent risk factor of atherosclerotic cardiovascular diseases (ASCVD)s and the cause of the disease (3,4). Current guidelines indicate that total cholesterol should be under 5 mmol/l and LDL-C under 3 mmol/l. For high risk patients, for example if atherosclerosis has been diagnosed, the target level for LDL-C is under 1.4 mmol/l (3).

ASCVD is initiated and further driven by the retention, modification, and aggregation of low density lipoprotein (LDL) particles in the arterial intima. Experimental work has demonstrated that aggregated LDL particles can induce lipid accumulation in macrophages and activate intimal cells and thereby induce inflammation in the arterial wall (5,6). Therefore in this thesis, I hypothesize that it is not only the plasma concentration of LDL-C that influences atherogenesis, but also the characteristics of LDL particles. This thesis aims at testing whether subjects with aggregation-prone LDL have an increased risk of developing coronary artery disease (CAD) and/or CAD deaths, and further if LDL the aggregation susceptibility is modifiable.

2 REVIEW OF THE LITERATURE

2.1 ATHEROSCLEROSIS

CVDs are the leading cause of death and disability around the world and also still in Finland despite intensive efforts at prevention and treatment of risk factors (1). Ischemic heart disease or CAD, carotid artery disease or cerebrovascular disease, peripheral artery disease (PAD) and chronic kidney disease are all classified as CVDs. ASCVD is a CVD that is specified as having been caused by atherosclerosis.

Sudden cardiac death is the cause of every second CAD death. Since most patients are never identified as high-risk patients prior to the event, it is evident that the prevention of these kinds of deaths is difficult (7-9). Most of the sudden cardiac deaths occur because of CAD, of which one third had not been diagnosed (7,8,10-12). The prevention of atherosclerosis focuses on the treatment of risk factors, but it seems probable that current treatment goals are not stringent enough, and/or that there are still unrecognised risk factors.

2.1.1 History

Leonardo Da Vinci was the first to describe an `old man` with the following words ”In the proportion as the vessels become old their branches lose their straightness and become so much the more bent or tortuous, and their coats thicker, as old age becomes full of years”. Around 1506, he also wrote that this `old man` died because of “failure of the artery that feeds the heart and lower members” (13,14).

Atherosclerosis is commonly assumed to be a modern disease, but atherosclerotic lesions have been detected already from members of pre-industrial populations including pre-agricultural hunter-gatherers who died 4000 years ago by studying their mummified remains (15). However, this still does not mean that atherosclerosis is normal or an inevitable part of human aging.

2.1.2 Definitions

Atherosclerosis is a disease of the arteries where the lumen of the artery narrows due to the formation of plaques, causing abnormal blood flow. Atherosclerosis starts to develop in childhood and its development usually continues for decades before it evokes any clinical symptoms (16-20).

In childhood, fatty streaks are the first visible sign of atherosclerosis, and they develop into intermediate lesions during adolescence and young adulthood (16-18). In an autopsy study, early stage aortic atheromas were found from all subjects examined at the age 15-19 years (n= 614) and over 50% had coronary lesions (19). Fibroatheromas and calcified lesions develop later in adulthood i.e. before the age of 50 years, 80% of men and 47% of women have calcified plaques in at least one artery (carotid, coronary, proximal, distal aorta or iliac vessels), and at 70 years of age, calcified plaques are ubiquitous in both men and women (n=650) (20).

The symptoms depend on the stage of the atherosclerotic disease and the diseased organ. Ischemic heart disease causes chest pain (angina pectoris), shortness of breath and arrhythmias, and may lead to myocardial infarction (MI), also known as heart attack. However, approximately 50% of cardiac deaths are sudden, without any previous symptoms (12). Carotid artery disease causes paralysis, loss of consciousness, headache and other neural symptoms, and may lead to stroke. PAD causes numbness, pain and intermittent claudication. Chronic kidney disease is associated with a slow loss of kidney function which is usually asymptomatic; symptoms appear in the late stage of the disease, for example swelling and loss of appetite.

The diagnosis of the ASCVD is based on clinical status and the results from several tests. The electrocardiogram (ECG) measures electrical activity of heartbeat, and changes in normal ECG indicate cardiac abnormalities. An exercise stress test can be used for stable patients to provoke changes in ECG. Imaging methods, including angiogram and a computer tomography scan can be used to visualize diseased arteries. These provide information about the severity of the disease and are used to choose the most appropriate preventative treatment strategies to avoid further cardiovascular events and deaths (3).

2.1.3 Risk factors and biomarkers

There some non-modifiable conventional risk factors for atherosclerosis i.e. advanced age, male gender, family history and genetic abnormalities. The modifiable risk factors include dyslipidemia, hypertension, glycemic control in diabetes, and insulin resistance.

Behavioural risk factors have crucial roles as risk factors for CAD, for example smoking, Western type diet, obesity, physical inactivity, and excessive alcohol consumption. Non-smoking, physically active people with healthy diet, moderate alcohol consumption and no abdominal adiposity have been found to display a 86% lower risk for primary MI in comparison to people who do not engage in these healthy behaviours (21). Dyslipidemia, hypertension, type 2 diabetes, and insulin resistance can all be managed by modifying these behavioural risk factors (3).

Dyslipidemia includes high total cholesterol, LDL-C, triglycerides (TGs), and low high density lipoprotein cholesterol (HDL-C). The recommended levels for LDL-C vary depending on the patient´s CVD risk category. In the general population, LDL-C is recommended to be <3.0 mmol/L (<116 mg/dL) but the value is much lower for very high risk patients i.e. LDL-C <1.4 mmol/L (<55 mg/dL) (3). The LDL-C concentration in the inner layer of the arterial wall, the intima, is similar to LDL-C concentration in plasma (22). The physiological plasma LDL-C concentration is low, around 1.0 to 1.5 mmol/l; when this limit is exceeded, LDL particles begin to accumulate into arterial intima (22-25). Despite the provision of intensive LDL-C lowering therapies, a residual risk remains (26).

Approximately every second patient who has concentrations of total cholesterol and LDL-C that are currently considered normal, and no other risk factors for CAD, still has calcified plaques when he/she reaches middle-age (27). It has been suggested that lower target levels for total cholesterol and LDL-C might more efficiently prevent atherosclerosis as well as the earlier adoption of lifestyle and medical interventions (28). Certain phenotypes of LDL, such as small dense LDL, oxidized LDL (oxLDL) and lipoprotein (a) (Lp(a)), are considered particularly pathogenic, thus the measurement of only LDL-C levels does not fully capture the LDL-related risk of ASCVD (29,30). In fact, additional measurements that are related to

the pathophysiological functions of LDL particles may help to unveil the actual ASCVD risk that is not explained by conventional risk factors.

Interest in finding novel biomarkers has been increasing, and recently plasma sphingolipids have been suggested to be related to atherosclerosis and cardiovascular deaths (31-34). Sphingolipids are molecules containing a sphingoid base, mostly 18 carbon-long with one double bond (C18:1 or simply 18:0) and a fatty acyl group. To allow an easier nomenclature of sphingolipids, the sphingoid base is usually left out, for example, ceramide 18:1/16:0 is ceramide (Cer) 16:0. Sphingomyelins (SM) and ceramides are subgroups of sphingolipids that are found in LDL particles and their structure will be discussed in chapter 2.3.3. "Lipoprotein structure". Bioactive sphingolipids play a role in inflammatory signalling and apoptosis (35) and they are enriched in atherosclerotic plaques (36).

Plasma Cer 18:0 and 18:1 levels have been shown to predict major cardiovascular events in population cohort (FINRISK 2002) (37). Similarly, the plasma Cer 18:1 concentration was found to associate with major adverse cardiac events and vulnerable plaques in the prospective ATHEROREMO IVUS-study (38). The LURIC study showed that plasma levels of Cer 16:0, 18:0 and 24:1, as well as plasma SM 16:0, 16:1, 24:1 and 24:2 concentrations associated with cardiovascular mortality. Similarly, the amounts of ceramides 16:0 and 24:1 were found to predict future CAD deaths in three independent prospective studies (Corogene, BECAC and SPUM-ACS) (31). The predictive power of these lipids became stronger if these lipids were divided with the level of Cer 24:0 (31). Recently, authors have improved their earlier ceramide risk score, which now includes only three ratios, Cer 24:1 to Cer 24:0, two ceramide ratios for phosphatidylcholines (PCs); Cer 16:0 to PC 16:0/22:5 and Cer 16:0 to PC 14:0/22:6, and one individual lipid, PC 16:0/16:0 concentration (39). At present, while there are some inconsistencies in the results, it does seem that at least some ceramides and SMs are indicators of an increased risk for ASCVD. The future will show which kind of lipids or lipid ratios are best for ASCVD risk estimation and may be beneficial in the development of novel treatment strategies.

Ethnicity is a genetic factor that has an effect on the ASCVD risk. For example, people originating from South Asia (India, Nepal, Bangladesh, Bhutan, Pakistan, and Sri Lanka)

have a higher risk for ASCVD than other ethnic groups (40). South Asians experience their first MI on average 10 years earlier and they have higher morbidity and mortality rates for ASCVD as compared to white Caucasians (41). South Asians have a higher prevalence of ASCVD risk factors and they have higher body fat percentage, however the high risk for ASCVD cannot be solely explained by these factors alone (42-44).

Risk factors are evaluated with laboratory tests and blood pressure measurement. In addition, calculating risk scores can help to predict the risk of coronary heart disease and stroke, e.g. the Systematic COronary Risk Evaluation (SCORE) or FINNRISK-calculator. For example, FINNRISK-calculator evaluates the percentual risk to develop CAD/ MI or stroke in 10 years. The prediction is based on age, gender, smoking, HDL-C, LDL-C, blood pressure, family history and possible diabetes. An elevation of the risk by 10% or higher is considered as a high risk for CAD/MI/stroke; for SCORE, the limit is 5%. In addition, polygenic risk scores will most probably become increasingly important in the future in the evaluation of risk to develop CAD as well as in the development of treatment strategies (45).

2.1.4 Treatment

When treating atherosclerosis, the management of modifiable risk factors is important. Non-medical interventions include smoking cessation, adoption of a Mediterranean diet or some other healthy diet, weight loss if overweight, exercise and reducing alcohol consumption. To reduce LDL-C concentration to target levels, statins are the primary medication (3). Statins have been demonstrated to prevent MI and cardiovascular deaths, both in primary prevention and after MI (46-51). Statins inhibit the β-hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis in the liver, which leads to an increase in the numbers of LDL-receptors (LDLR) on the surface of the liver, and reduced total and LDL-C concentrations in plasma. Ezetimibe can be an alternative or additive treatment to reduce LDL-C concentrations, if statins are not suitable or if target levels cannot be reached with statins alone (52,53). Ezetimibe prevents the dietary and biliary cholesterol absorption in the intestine (54). Since, less cholesterol is transported to the liver, similarly to the situation with statins, this leads to a reduction in intracellular cholesterol which promotes upregulation of LDLR expression, and thus reduces the plasma LDL-C level. Proprotein convertase subtilisin-kexin type 9 (PCSK9) monoclonal antibodies

(mAb)s are new group of medicines that lower LDL-C dramatically; they are more effective in preventing CAD events when combined with statins than can be achieved with only statins alone (3,55-57). Circulating LDL particles bind to LDLRs on the surface of the liver, LDLRs are transported with LDL particles inside the hepatocytes, where LDL is released and LDLR is transported back to the surface of the liver. Plasma PCSK9 can bind to LDLR, which is transported to lysosomes where it is degraded, which lowers the amount of LDLRs on the surface of the liver, and increases the plasma LDL-C concentration (56). If PCSK9 antibody binds to PCSK9 in plasma and prevents a PCSK9-LDLR interaction in hepatocytes, LDLR is transported back to the surface of the liver, and the plasma LDL-C concentration declines.

It has been estimated that a reduction of LDL-C by 1 mmol/l reduces major vascular events by 21% (58). Statins can reduce LDL-C by 20-40% depending both on which statin is administered and its dose (59). The most effective treatment to reduce LDL-C can be achieved when statins are combined with PCSK9 mAbs, resulting on average of an over 50% further reduction in LDL-C (60,61) while ezetimibe combined with statins lowers LDL-C only by a further 24% (62).

2.2 ATHEROGENESIS

2.2.1 Historical view

In 1910, Adolf Windaus, together with Karl Albert Ludwing Aschoff, observed that that human atherosclerotic aortas contained over 20-fold more cholesterol esters than normal aortas (63), Windaus was awarded a Nobel prize in 1928 for his scientific work with sterols. In 1913, Anitschkow and Chalatow demonstrated that feeding of rabbits and guinea pigs with a diet high in cholesterol or egg yolk induced lipid deposits in the arteries (64).

2.2.2 Response-to-Retention hypothesis

`Response-to-Retention hypothesis` is the leading theory for atherosclerotic plaque formation and it is supported by a wealth of scientific evidence (65,66). In addition to the Response-to-Retention hypothesis, there are two other hypotheses; Response-to-Inflammation (67) or Response-to-Injury (68). The former highlights the role of low-grade inflammation in the artery wall, and the latter states that the initial event in atherogenesis is an injury of the endothelium. However, all these hypotheses are linked to each other, for example one of the first steps is invariably a lipid influx into the artery wall. Apolipoprotein B (apoB) containing LDL, small very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and even small chylomicron remnants smaller than 70 nm can cross an intact endothelium and reach the tunica intima (69). Endothelial dysfunction enhances lesion formation, and it occurs most commonly at arterial curves and branches, where blood flow is disturbed; these are called atherosclerotic prone areas (70,71).

The Response-to-Retention hypothesis suggest that retention or accumulation of apoB containing cholesterol-rich lipoprotein within arterial wall is the key initiating event in atherogenesis (**Figure 1**). Retained lipoproteins are modified in the intima by oxidation, as well as by proteolytic and lipolytic enzymes (66,72). The retention of the LDL particles in the intima depends both on the plasma LDL-C concentration and the features of the proteoglycans (73,74). These retained and modified lipoproteins trigger local inflammation attracting an influx of monocytes which subsequently differentiate into macrophages (5,6). Macrophages and smooth muscle cells avidly take up these lipoproteins leading to the

formation of foam cells and they also start to secrete proinflammatory mediators and enzymes (75). For example, $CD4^+$ T cells are also recruited into the developing plaque (76). Over time, a more advanced lesion develops, having activated macrophages, T-cells and mast cells that induce local inflammation. Complex plaques are covered by a fibrous cap and have a necrotic core formed from cell debris, necrotic and apoptotic cells and cholesterol crystals. This progress from harmless "fatty streaks" to a destructive "vulnerable plaque" is described below.

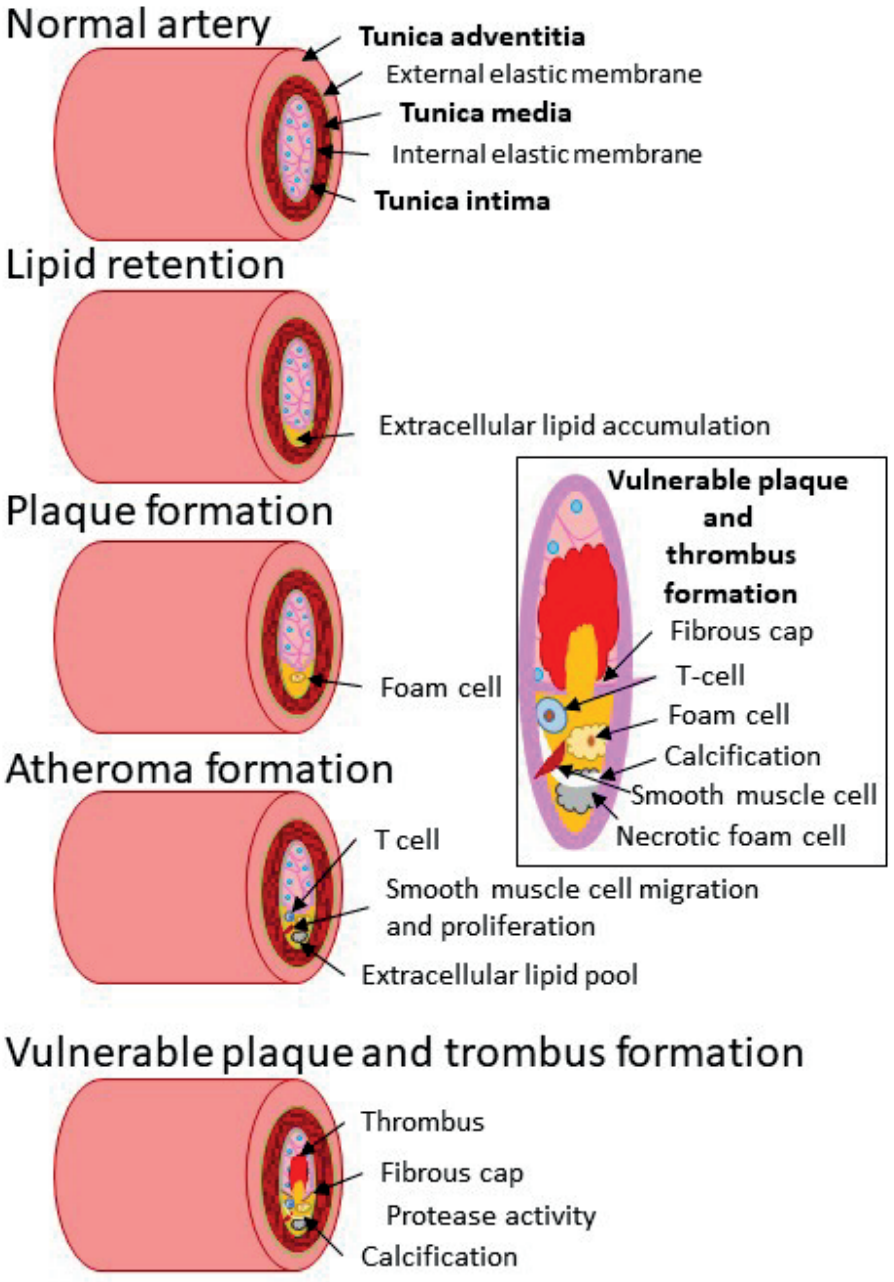

Figure 1. Atherogenesis. LDL accumulates under the endothelial layer in tunica intima. Fatty streaks are visible to the naked eye and when they have developed into atheromas, plaques can rupture, causing thrombosis or blocking circulation in artery. The insert shows a vulnerable plaque and thrombus formation.

2.2.3 LDL binding to arterial proteoglycans

Circulating LDL can enter the inner layer of the arterial wall, the intima, where LDL can bind to intimal extracellular proteoglycans leading to LDL retention (73,74,77). ApoB-100, the major apolipoprotein present in LDL particles, has segments of positively charged residues, lysine and arginine, that bind to the negatively charged carboxylic acid and sulfate groups of glycosaminoglycan chains of proteoglycans (78). Proteoglycans are proteins to which glycosaminoglycan chains are covalently attached and are a major component of the extracellular matrix.

Glycosaminoglycans are bound to the core protein, for example in the case of versican, it can further bind via a linker protein to hyaluronic acid, forming large components in extracellular matrix. (**Figure 2)**.

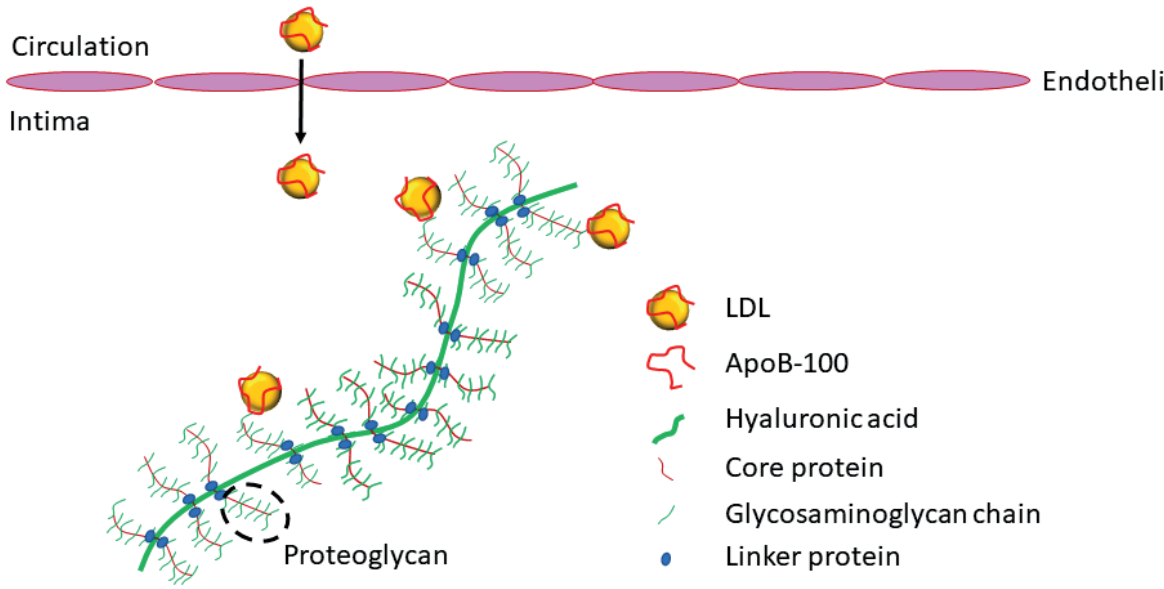

Figure 2. Structure of proteoglycan-hyaluronic acid complex and LDL binding to proteoglycans. LDL can bind to intimal extracellular proteoglycans via an apoB-100 – proteoglycan interaction.

2.2.4 LDL oxidation

When LDL particles are retained in the intima, they are exposed to modifying agents and enzymes. LDL can be oxidized by enzymatic or non-enzymatic catalysts, in a process involving reactive oxygen species (ROS). There is constant ROS production due to normal cell function and xenobiotic metabolism as well as due to the presence of pollutants, tobacco smoke and ionizing radiation etc. Thus, oxLDL causes endothelial dysfunction that has further pro-inflammatory and pro-atherogenic consequences but its concentration can be reduced by statin therapy (79,80). Minimally oxidized LDL particles can be recycled back to the circulation, and therefore the oxLDL concentration can be measured from plasma samples. The circulating oxLDL level is higher in patients having CAD and it is considered a biomarker for the disease (81). Instead, extensively oxidised LDL particles are recognized by scavenger receptors of intimal macrophages leading to foam cell formation (80).

2.2.5 LDL aggregation

In the intima, LDL particles are exposed to lipolytic, proteolytic and oxidative enzymes and agents. These modifications have a critical role in atherogenesis. Modified LDL particles are susceptible to aggregation and aggregated LDL particles cannot diffuse back to the circulation. LDL aggregates bind intimal proteoglycans more strongly than native or modified, non-aggregated LDL particles (82). LDL aggregation can be induced *in vitro* by various enzymes or oxidative agents, most commonly by acid sphingomyelinase (SMase), neutral SMase, phospholipase A_2 (PLA_2), α-chymotrypsin, or $CuSO_4$ oxidation. Of these, only acid SMase forms large LDL aggregates (>3,000 nm), whereas even after overnight incubations, other agents form only small LDL aggregates (30-200 nm) (83). In the arterial intima, acid SMase can hydrolyse LDL-SM to ceramide, which can lead to LDL aggregation and promote the initiation and progression of atherosclerosis (36,84,85). Indeed, human atherosclerotic plaques have been demonstrated to contain aggregated lipoprotein particles (86,87) and particularly the largest aggregates are enriched in ceramides, the lipolytic product of SM hydrolysis by SMase (36,88). In rabbits, it has been shown that after only two hours of bolus injection of human LDL, LDL can accumulate in intima, but also forms LDL aggregates in focal areas of the intima (36). Because LDL can become aggregated very rapidly in the intima after injection into the circulation, oxidation is not likely to be the reason

for this fast *in vivo* aggregation and arterial SMase has been shown to hydrolyze LDL-SM (36), and thus SMase is *in vivo* one of the most potent cause of LDL aggregation.

Furthermore, aggregated LDL particles promote extensive foam cell formation (89,90) by mechanisms that have been suggested to involve the catabolism of aggregated LDL in the extracellular acidic hydrolytic compartment, a process observed both *in vitro* and *in vivo* (91,92). Aggregated LDL particles have also the potential to induce inflammation in the intima and to promote plaque destabilization (87,93). LDL modified by the combination of protease and cholesteryl esterase releases free fatty acids (FFA)s, of which oleic acid and linoleic acid particularly induce interleukin (IL)-8 secretion from endothelial cells (94). Furthermore, IL-8 triggers adhesion and migration of circulating monocytes into the intima (95).

2.2.6 Inflammation in atherogenesis

Human atherosclerosis is driven by lipid accumulation, which leads to chronic local inflammation of arterial intima. The modification of any apoB-containing lipoprotein can induce local innate and adaptive immune responses in the intima (96,97). The lipoproteins can activate inflammatory cells in the intima, after which these cells start to secrete pro-inflammatory cytokines and chemokines that activate both endothelial cells and circulating leucocytes (98,99). This results in the further recruitment of inflammatory cells of myeloid origin, especially monocyte migration into the intima. In the intima, the monocytes become transformed into macrophages that secrete enzymes and agents having the potential to modify lipoproteins (100). Cellular activation and lipoprotein modification lead to the creation of a vicious cycle, in which modified lipoproteins can provoke cells to secrete higher amounts of enzymes and agents to further modify lipoproteins. Finally, foam cells undergo some form of cell death, releasing their lipid contents into the extracellular fluid, and if removal of cell debris by new macrophages does not occur, this can lead to the formation of a mature atherosclerotic plaque with a necrotic core (101).

Pro-inflammatory macrophages activate adaptive immunity mainly by helper T cells ($CD4^+$). Antigen presenting cells can activate helper T cells by presenting LDL-derived peptides and further T cells activate pro-inflammatory pathways in the plaque niche (102). In addition,

many other immune cells are known to be present in human atherosclerotic plaques, e.g. natural killer cells, mast cells, and eosinophils (103).

2.3 LIPOPROTEIN / LDL METABOLISM

2.3.1 Historical view

Konrad Bloch and Feodor Lynen were awarded the Nobel Prize in Physiology and Medicine in 1964 "for their discoveries concerning the mechanism and regulation of the cholesterol and fatty acid metabolism." In 1971, the Framingham prospective cohort study associated serum cholesterol with increased risk of cardiovascular disease (104). Michael Brown and Joseph Goldstein were awarded the Nobel Prize in Physiology or Medicine in 1985 “for their discoveries concerning the regulation of cholesterol metabolism”.

2.3.2 Lipoprotein structure

The purpose of lipoproteins is to transport hydrophobic lipid molecules in hydrophilic fluids, such as blood, lymph or extracellular fluids. Lipoproteins consist of hydrophobic core lipids with an amphipathic surface monolayer of lipids, and apolipoproteins. The major lipoproteins are described in **Table 1**, including their main core lipid composition and apolipoproteins attached to each lipoprotein. The hydrophobic core lipids are triacylglycerols (TG)s and cholesteryl esters (CE)s (**Figure 3**); the ratio of these compounds depends on the lipoprotein particle. The surface lipids are mainly phospholipids and unesterified cholesterol (UC). Phospholipids are further classified as PCs, SMs, and their hydrolysis products, lysophosphatidylcholines (LPC)s and ceramides (**Figure 4**). In addition, minor lipids, for example phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) are found in these particles.

Table 1. Human lipoproteins and their main core lipid and apolipoproteins. Very low density lipoprotein, VLDL, low density lipoprotein, LDL, intermediate density lipoprotein, IDL, lipoprotein (a), Lp(a), high density lipoprotein, HDL. Modified from (105).

Proatherogenic lipoproteins							**Anti-atherogenic lipoproteins**	
	Exogenous lipid transport system		Endogenous lipid transport system					
Particle	**Chylomicron**	**Chylomicron remnant**	**VLDL**	**VLDL remnant (IDL)**	**LDL**	**Lp(a)**	**HDL**	**Discoidal nascent HDL**
Radius (nm)	1000-80	100-50	70-30	30	20	20	8.5-10	5
Major core lipid	TG	CE	TG	TG	CE	CE	CE	no core
Major apolipo-proteins	A B-48 C E	A B-48 E	B-100 C E	B-100 E	B-100	B-100 a	A C E	A
Site of synthesis	intestine	circulation	liver	circulation	circulation	liver	intestine circulation liver	

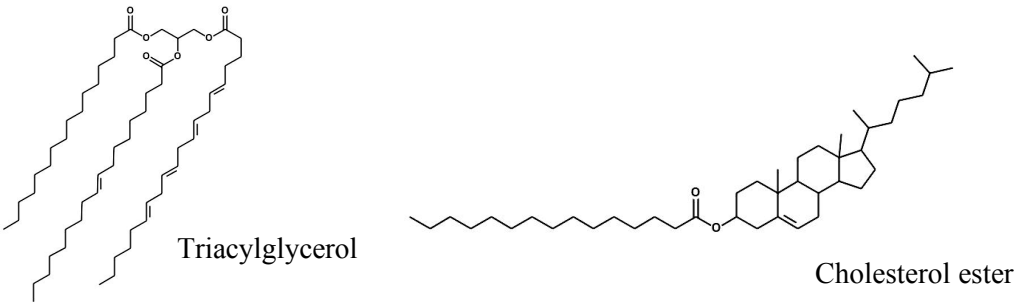

Figure 3. LDL surface lipid structures. Triacylglycerides have a glycerol backbone and three ester- linked fatty acid chains. This triacylglycerol has a saturated fatty acid, palmitate (18:0) at sn1-position, a monounsaturated oleic acid (18:1) at sn2-position, and a polyunsaturated arachidonic acid at sn3-position. Cholesterol esters have one fatty acid linked to cholesterol with an ester bond.

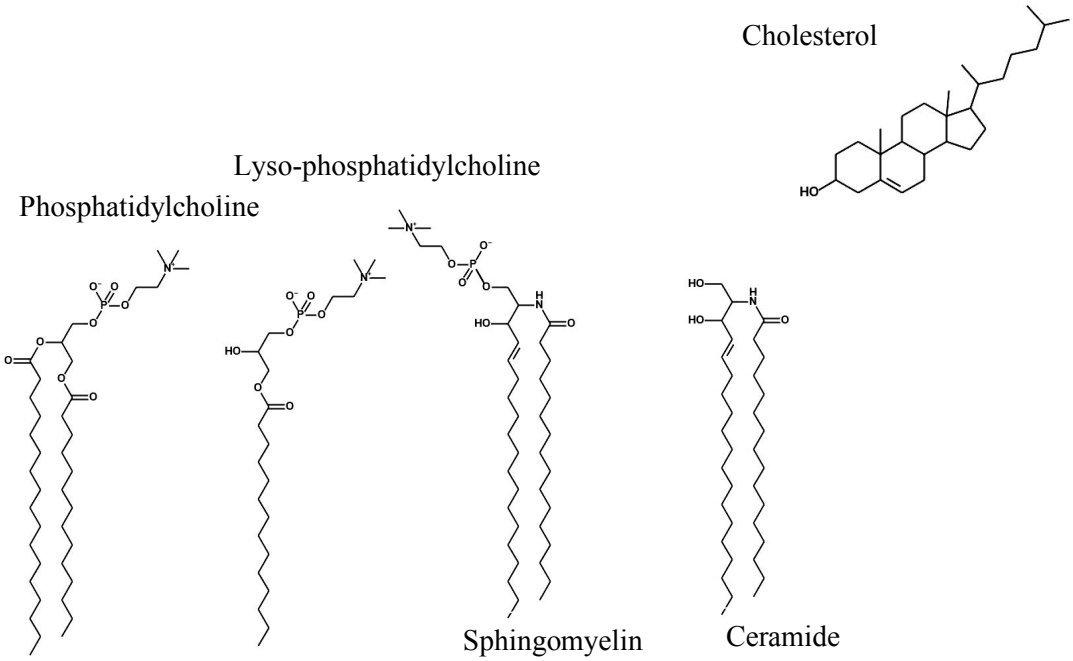

Figure 4. **LDL surface lipid structures.** Cholesterol has a hydrophilic hydroxyl group (-OH), four hydrophobic steroid rings and a hydrocarbon tail. Phosphatidylcholines have a glycerol backbone and two ester-linked fatty acids and a hydrophilic phosphocholine head group. Lysophosphatidylcholines have a similar structure, but they have only one fatty acid linked to glycerol. Sphingomyelins have a sphingosine backbone, a phosphocholine head group and one fatty acid linked to it via an amide bond. Sphingomyelinase can break down sphingomyelin to ceramide and phosphocholine.

2.3.3 LDL particle structure

The LDL particle structure is described in **Figure 5**. Like other lipoproteins, LDL particles have a hydrophobic core that in the case of LDL, is rich in CEs. The major component of LDL particle is CE (~40%, wt/wt), followed by phospholipids (PL) (~20 %), apoB-100 (~20%), UC (~10%) and TGs (~5%). The surface monolayer of LDL particles contains mainly two types of phospholipids; about 450 molecules of PC and about 185 molecules of SM. Two-thirds of about 600 molecules of UCs are located on the surface and one-third in the core. The LDL particle surface also contains small amounts of other lipids, including LPC (about 80 molecules), PE (about 10 molecules), ceramides (about 2 molecules) and some PI (106). The principal structural apolipoprotein of LDL is a single copy of apoB-100 that surrounds the particle and interacts with surface lipids. The core of the particle consists predominantly of CEs (about 1600 molecules) and a relatively small amount of triglycerides (about 170 molecules) (106).

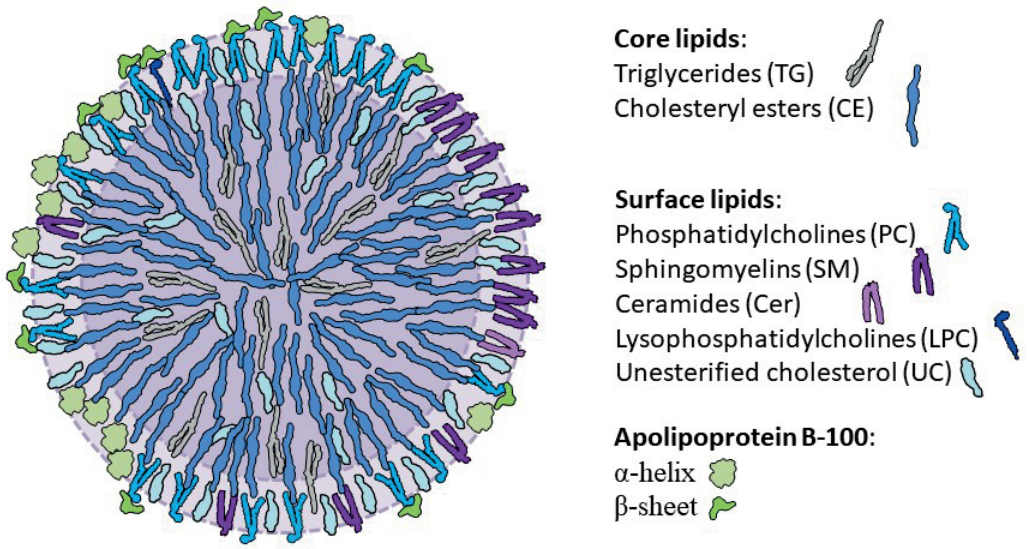

Figure 5. LDL structure. LDL particle has a hydrophobic core and amphipathic surface lipids. A single copy of apolipoprotein B-100 (apoB-100) surrounds the particle. ApoB-100 has domains rich in amphipathic α-helices and β-sheets. Modified from (106).

2.3.4 Exogenous lipid metabolism

Almost all i.e. 95%, of the dietary fats are triglycerides (TGs), the remaining 5% are phospholipids (PLs), CEs, FFAs and fat-soluble vitamins. Dietary TGs are converted in stomach and duodenum by gastric lipase to monoglycerides and FFAs, similarly CEs are de-esterified to UC and FFAs.

FFAs, monoglycerides and UC form micelles with bile acids, and can then be absorbed from intestine into enterocytes. In enterocytes, these lipids are re-esterified and packaged into chylomicrons. Chylomicrons are the largest lipoproteins, having one apoB-48 protein on the surface of the particle. TGs and CEs form the hydrophobic core with PLs and UCs as the hydrophilic monolayer on the surface of the lipoprotein, where the apolipoprotein is attached. Chylomicrons are then transported via the lymphatic system into the circulation. ApoC-II proteins from VLDL and HDL become attached to chylomicrons in the circulation and they activate endothelial lipoprotein lipase (LPL) in muscle and adipose tissue capillaries (107). LPL hydrolyses TGs to FFAs and glycerol that are used for energy production or storage (108). The majority i.e. 70-80%, of chylomicron-TGs are hydrolysed with a few minutes after they gain access to the circulation. As the core becomes smaller, the surface material of chylomicrons folds and is attached to HDL particles. The lipolyzed 10-fold smaller chylomicron remnants lose apoC-II with this being replaced by apoE from other apolipoproteins. ApoE is recognized by LDLR of hepatocytes and chylomicron remnants can therefore be taken up into the liver (109).

In hepatocyte lysosomes, chylomicron remnant lipids are hydrolysed to FFAs, glycerol and UC; apolipoproteins are also hydrolysed to amino acids. UC is then transported to the cytosol, from where it is secreted to bile fluid as a UC or bile acids. Almost all (95%) of the bile acid secreted to the intestine will be recycled back to the liver through the enterohepatic circulation (110). In contrast, UC absorption from intestine varies between individuals from 25 % to 80 % (111). Because cholesterol cannot be hydrolysed in human cells, the only excretion route is via the intestine as bile acids (one third) or as UCs (two third) to faeces (112).

2.3.5 Endogenous lipid metabolism

Hepatocytes produce VLDL particles that transport TG and CE back to the circulation. VLDL particles has typically three types of apolipoproteins, apoB-100, apoE, and apoC-II, and the core is rich in TGs as compared to CEs. VLDL metabolism is similar to chylomicrons, TGs are lipolyzed by LPL and as the core becomes smaller, cholesteryl ester transfer protein (CETP) moves CEs from HDL to VLDL (113). ApoC-II is released from the surface, the core is enriched in CEs, and the particle is now called a VLDL remnant or an intermediate density lipoprotein (IDL). VLDL remnants can be taken back into the liver by LDLRs that recognize both apoB-100 and apoE. Approximately 50% of VLDL remnants are taken into the liver and the other half rapidly lose their apoE, such that the core consists now mostly of CEs, and the particle is called LDL (114). (**Figure 6**)

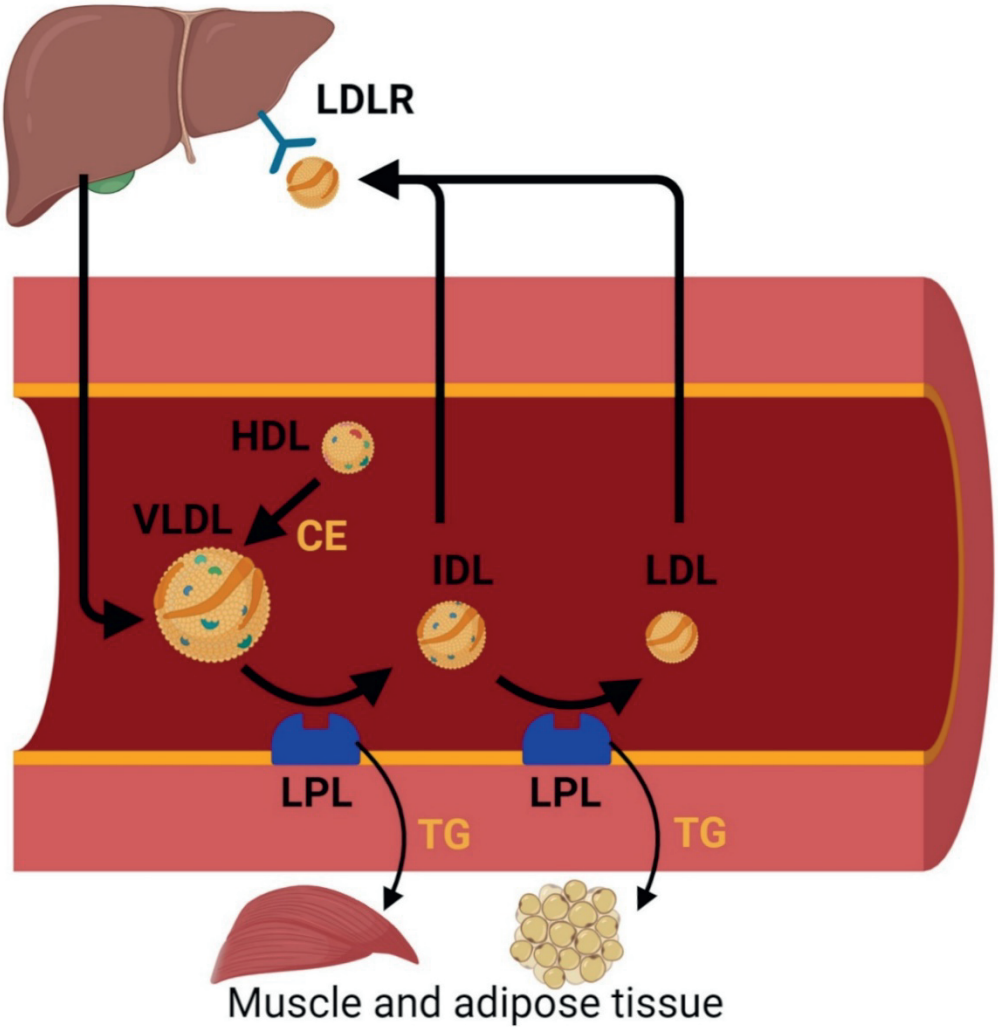

Figure 6. Endogenous lipid metabolism. Very low lipoprotein particles (VLDL) are produced in the liver and secreted into circulation. Lipoprotein lipase (LPL) hydrolyses triglycerides (TG)s from VLDL particles and the particle core becomes smaller. TGs are used for energy production in muscle tissue or stored in adipose tissue depending on the metabolic state. Cholesteryl ester transfer protein (CETP) transports cholesterol esters (CE)s from high density lipoprotein (HDL) particles to VLDL. The core becomes enriched with CE and is called an intermediate density lipoprotein (IDL). IDL further loses TGs and it becomes a low density lipoprotein (LDL) particle. IDL and LDL particles can be captured by the liver via the LDL-receptor (LDLR) or they can be taken up by the peripheral tissues. Figure was created with BioRender.com

Most of LDL particles are taken back to the liver by apoB-100 - LDLR interactions or hepatic scavenger receptors, the remaining 30 % are taken up by extrahepatic tissues. Extrahepatic cells can take up LDL via the LDLR and hydrolyse CE to UC that can be used as a structural component e.g. for the synthesis of biological steroids (115). LDL can be taken up into peripheral tissues and in normal circumstances, excess cholesterol is transported to the lymphatic system from where it is passed back into the circulation (116). All cells in the human body have the capability to produce cholesterol.

Lp(a), is LDL that contains apoprotein (a). Apo(a) is a large glycoprotein, having a high homology with plasminogen. Because of this homology, Lp(a) inhibits fibrinolysis, by competition with plasminogen binding, and thus it may increase blood clotting (117,118). However, a recent study revealed that *ex vivo* clot analysis was not affected by Lp(a) (119). Lp(a) is an independent risk factor for CAD, and it is not effectively reduced by LDL-C lowering medication (120).

HDL particles have the ability to take cholesterol from cells back to the circulation and the liver, in a process called reverse cholesterol transport. HDL metabolism is complex, and a heterogeneous group of HDL particles have been identified. HDL can bind to scavenger receptor class B type 1 (SR-B1) receptors in the liver, or transfer cholesterol to apo-B containing proteins by CETP (114).

2.4. NUTRITION AND LIPID METABOLISM

2.4.1 Historical view

The diet-heart hypothesis suggests that reducing dietary fats reduces plasma cholesterol, and thereby reduces the ASCVD risk. The hypothesis was proposed by Ancel Keys in the 1950s and in 1980s he published the results from Seven countries study revealing the clear correlation between dietary saturated fats and coronary mortality (121). In 1968, it was thought that dietary cholesterol, which is present in high amounts in foods containing saturated fats, increased plasma cholesterol, and was the main dietary risk factor for atherosclerosis (122). The hypothesis has been modified since then from total saturated fat or cholesterol consumption to a recommendation of replacing saturated fats with polyunsaturated fats (PUFAs) (3,123,124).

2.4.2 Dietary components and atherosclerosis risk

It is estimated that dietary factors are linked to 11 million deaths (22 % of all deaths) every year and 255 million disability adjusted life years (15% of all disability adjusted life years) (125). Diet is also an important part of prevention and treatment of ASCVD, since it a good way to modify risk factors of the disease (126,127). Replacing dietary saturated fats with polyunsaturated fats has been shown to decrease total cholesterol, LDL-C, and triglyceride concentration in plasma and the incidence of major cardiovascular events (128-130). Replacing saturated fat with polyunsaturated fats, predominantly linoleic acid and α-linolenic acid, or with monounsaturated fats, predominantly oleic acid, is more favourable than replacing saturated fat with carbohydrates (128). Polyunsaturated fats have the most beneficial effect on total and LDL-C (128). The Prospective Urban Rural Epidemiology (PURE) study disputed the harmfulness of saturated fats (131), and this triggered a lively discussion. In the PURE study, the authors concluded that total fat intake had no effect on cardiovascular health or even that the effect might be beneficial, because they had found an association between higher intake of saturated fats and a lower risk for stroke and total mortality (131). The PURE study has been widely criticised, the authors did not qualify the type of carbohydrates and the majority of the study subjects came from low-income

countries, where the diet consists of white rice and many of the participants suffered from undernutrition. (132,133). The conclusions of the PURE study were based on baseline dietary information, collected from food frequency questionnaires, that are known to underestimate food intake, especially fat intake (134).

From a mechanistic standpoint, it is known that PUFAs reduce VLDL secretion from liver because they are more prone for oxidation, which leads to the degradation of apoB-100 (135). After these discussions, current recommendations still emphasize that saturated fats should be avoided and replaced with polyunsaturated fats (3,123,124).

The diet-heart hypothesis originally postulated that dietary saturated fats and cholesterol increase plasma cholesterol levels and the risk for CAD (121,122). The hypothesis is no longer so straightforward, the inter-individual variations in how much cholesterol is absorbed from the intestine and feedback response of endogenous cholesterol synthesis have divided individuals into hyper-responders and hypo-responders to dietary cholesterol (136). The dietary cholesterol intake is recommended to be less than 300 mg/day, especially among individuals with high plasma cholesterol levels (3).

The dietary fatty acid composition directly affects the plasma fatty acid composition. For example, a high intake of n-3 PUFAs, such as fatty fish (containing eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA) increases the levels of these PUFAs in plasma CEs, TGs and PLs; the consumption of *Camelina sativa* oil (containing α-linoleic acid), increases plasma α-linoleic acid in plasma CEs, TGs and PLs (137).

A number of randomized, placebo controlled, double blind clinical trials have shown that food products supplemented with plant stanol esters can lower plasma LDL-C levels on average by 10-15 % if there is a 2-3 g/day plant stanol intake (138,139). This kind of decrease in LDL-C achievable by eating a plant stanol ester enriched spread is as effective in reducing estimated cardiovascular risk as adherence to the Mediterranean diet (140). Plants do not contain any cholesterol, but they have phytosterols; plant stanols and sterols. There is a small quantity of plant stanol esters in the human diet with their main source being whole-grain foods, and some amounts are also present in other foodstuffs, for example in vegetable oils, fruits, vegetables, and nuts (141). Chemically, these sterols/stanols are very similar to

cholesterol **(Figure 7)**, but in the human body, they act very differently. While 25 to 80 % of cholesterol is absorbed from the intestine, plant sterols are hardly absorbed at all, only about 0.5 % and plant stanols, even less i.e. by 0.05% (142). Plant stanols and sterols have a dual effect in the intestine, they reduce both dietary cholesterol absorption and bile cholesterol reabsorption. The daily intake of plant stanols in the Finnish population is relatively small, for plant stanol esters, it is 24 mg/day for men and 17 mg/day for women, and for plant sterols about 305 mg/day and 237 mg/day (141). Thus a biologically effective dose (2-3 g/day) can only be achieved with products enriched with phytosterols, most commonly plant stanol-enriched spread (3).

When it comes to diet, if the individual leaves something out, then it needs to be replaced with something else. As stated earlier, the most beneficial effect can be achieved by substituting saturated fats with unsaturated fats. (3,123,124) The other macronutrients which can replace saturated fats are carbohydrates and proteins. Carbohydates, especially a high intake of sugars, like fructose, increases plasma LDL-C, TG and decreases HDL-C (143-145), by increasing *de novo* lipogenesis. The regular consumption of sugar-sweetened beverages in women is associated with a higher risk for MI (146). Consumption of high or low carbohydrate diets are both unhealthy, as also low carbohydrate, high fat diets have been shown to increase LDL-C (147). Similar to the situation in carbohydrates and fats, also in proteins, quality matters. Plant based-protein and low carbohydrate diets are associated with the lowest CVD mortality (148), while animal based-protein and low carbohydrate diets have been linked with higher CVD and CVD mortality (148,149).

Sodium intake is associated with blood pressure and CAD risk (150,151). The majority of dietary salt comes from processed foods, and thus the consumption of unprocessed foods is recommended (123). In contrast, dietary potassium has beneficial effects on blood pressure and CAD risk (152). The main source of potassium in diet is fruits and vegetables, and a high consumption of these foodstuffs is recommended (123).

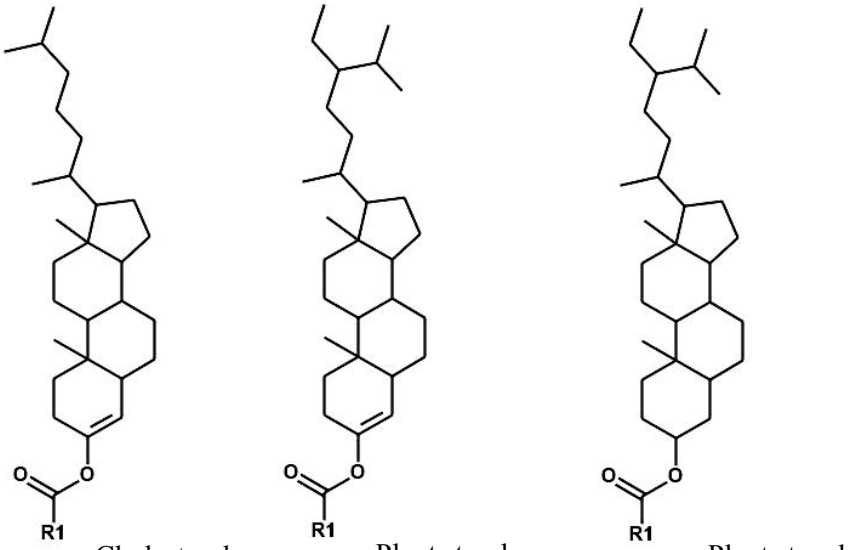

Figure 7. Chemical structure of cholesterol ester, plant stanol ester and plant sterol ester. Cholesterol is produced in humans, whereas plant sterol esters and plant stanol ester are synthesized only in plants.

The consumption of several vitamins has been associated with reduced CAD risk in observational studies, with most evidence for vitamins A, E and D. However, interventional trials have failed to confirm these findings, and, in contrast, have even suggested that supplemented vitamins A and E are harmful (153,154). Therefore, there are no recommendations of vitamin supplementations for CVD prevention (123). A higher dietary fibre intake is associated with a lower risk for CVD and therefore a higher intake of fibre is recommended (123,155).

2.4.3 Foods, dietary patterns and atherosclerosis risk

People eat food, not macronutrients, which makes it hard to demonstrate causal associations between nutrients and disease risk. The Mediterranean diet has been the most extensively studied as a dietary pattern to prevent CVDs (156). It has been shown to decrease total cholesterol levels and the CVD risk in both Southern European (157) and Eastern European populations (158). The Prevención con Dieta Mediterránea (PREDIMED) trial indicated that participants assigned to a Mediterranean diet, supplemented with extra olive oil or nuts had

a significantly lower incidence of major CV events compared with those who were on a control (low-fat) diet, with a relative difference of 30% (130,157,159). The Scandinavian alternative to the Mediterranean diet is the healthy Nordic diet that includes whole grains, fruits and vegetables, berries, low-fat dairy products and fatty fish (160). The healthy Nordic diet has been associated with a reduction in blood pressure, LDL-C levels, and obesity in type 2 diabetic patients (161) as well as in the prevention of strokes (162).

Both Mediterranean and healthy Nordic diet include a limited intake of red meat. A very recent study suggested that a higher consumption of red and processed meat would be associated with a higher ischemic heart disease risk, whereas higher consumption of cheese, eggs, and yogurt exerted an inverse effect (163). Plant products were not analysed in that study, and they may have beneficial effects as compared to animal products (164). Since both meat and cheese are high in saturated fats, the authors speculated that the difference could be attributable to the level of carnitine in meat; this quaternary amine can be metabolized to trimethylamine oxide, and has been shown to associate with an increased risk of atherosclerosis (163,165).

3 AIMS OF THE STUDY

This study aims to define LDL aggregation susceptibility as a novel modifiable and measurable factor associated with ASCVD risk, including interventions to affect LDL aggregation susceptibility.

1. To develop a method to measure the LDL aggregation susceptibility from plasma samples. (I)
2. To study if the lipid composition of LDL particles mechanistically explains the inter-individual variance in the LDL aggregation susceptibility. (I)
3. To examine if the LDL aggregation susceptibility is associated with atherosclerosis and if it predicts future cardiovascular deaths. (I)
4. To investigate if ethnicity affects the LDL aggregation susceptibility in an Ethnicity study investigating subjects with either South Asian or white Caucasian origins. (II)
5. To explore if the healthy Nordic diet or extra calories from saturated fat, unsaturated fat or carbohydrates affect the LDL aggregation or other quality properties of LDL. (III)
6. To investigate if PCSK9 monoclonal antibody treatment exerts a beneficial effect on the LDL aggregation susceptibility. (I)

4 MATERIALS AND METHODS

4.1 PATIENT COHORTS

The patient cohorts included in this thesis are presented in **Table 2.** and described in more detail below.

Table 2. Patient cohorts.

Cohort *Official Title*	*Study type, Time Perspective*	*Ref.*	*Clinical trial nro.*	*Study*
Population cohort, n=100 *Health 2000 Health examination study*	Epidemiological	(166)		I
Healthy Nordic diet intervention, n=57 *The Effect of Nordic Recommended Diet on the Features of Metabolic Syndrome* - Multicentre Study (SYSDIET)	Interventional, Randomized, Parallel Assignment	(167)	NCT00992641	I
Patients with coronary stenosis, n=48 *Genetic Predisposition of Coronary Artery Disease* - The COROGENE Study	Observational, Prospective	(168)	NCT00417534	I
Phase ll trial for PCSK9 monoclonal antibody, n=40 *Study of the Safety and Efficacy of MPSK3169A in Patients With Coronary Heart Disease or High Risk of Coronary Heart Disease* -The EQUATOR Study	Interventional, Randomized, Parallel Assignment, Double blinded Placebo-controlled	(169)	NCT01609140	I

Ethnicity study, n=24 *Exenatide and Brown Adipose Tissue (exe01)*	Interventional, Single group Assignment, no blinding	Unpub	NCT03002675	II
Hypercaloric diet, n=36 *Effects of Overfeeding Followed by Weight Loss on Liver Fat Content and Adipose Tissue Inflammation*	Interventional, Randomized, Parallel Assignment, no blinding	(170)	NCT021333144	III

4.1.1 Health 2000 Health Examination Survey

Health 2000 survey is a large Finnish epidemiological survey, carried out from fall 2000 to spring 2001 by the National Public Health Institute (166). The total survey included 8028 participants, representing the Finnish population. Samples from 100 participants, 50 % male and 50 % female, aged 33 to 48 years that were largely healthy when examined in this study. The clinical characteristics for these 100 participants are presented in Study I, Table 1.

4.1.2 SYSDIET study

SYSDIET study was randomized, controlled, interventional, parallel assignment study performed in six centres: Kuopio and Oulu (Finland), Lund and Uppsala (Sweden), Aarhus (Denmark) and Reykjavik (Iceland) (167). The participants had features of metabolic syndrome without diabetes and they were randomized to eat either Healthy Nordic diet or Control diet. All the participants were obese (body mass index, BMI 28-35 kg/m^2) and had two of the other international diabetes federation criteria for metabolic syndrome (171). The Healthy Nordic diet included fruits and vegetables, wholegrain products, berries, rapeseed oil, low-fat dairy products and fish three times per week. The control diet was based on the average Nordic diet. The intervention lasted for 18-24 weeks. The study included total of 166 individuals that completed the study, of which 55 samples from Finland were available for this study; 33 samples from the Healthy Nordic diet group and 24 samples from the Control group, from baseline and the end of the intervention.

4.1.3 Corogene study

Corogene study was an observational, prospective study investigating Finnish patients with CAD (168). The study examined a total of 5295 patients and the samples were collected from summer 2006 to spring 2008. Coronary angiography was performed in all patients and blood samples were taken. For this study, 48 samples from nested case-control sub-cohort of the Corogene study (31) were available, these included data from the national death certificate registry. The patients in this study were all male, non-diabetic, and having at least 50 % stenosis in their coronary arteries. Twenty-four of these samples were from patients that had died during an average follow-up period of 2.5 years, and matching controls (n=24) were chosen from the group that experienced no cardiovascular events during the follow-up time.

4.1.4 EQUATOR study

EQUATOR study was a randomized, double-blind, phase 2 clinical study to investigate a fully human immunoglobulin G1 PCSK9 mAb (RG7652, Genentech, South San Francisco) (169). The study was performed from spring 2012 to summer 2013 in 9 countries. PCSK9 mAb or placebo was subcutaneously injected once, and samples were collected before and 29 days after the treatment. The study originally included 248 patients but samples from 40 patients were used in this study (PCSK9, n=25 and placebo, n=15).

4.1.5 Ethnicity study

This study was a part of a clinical trial which had been performed in the Netherlands from fall 2016 to spring 2018. (Janssen & Nahon et al., in preparation) All the study participants were healthy Dutch males, 12 white Caucasians and 12 South Asians. The South Asian participants had all 4 grandparents of South Asian descent. Plasma samples were collected after overnight fasting and body composition was measured.

4.1.6 Hypercaloric diet study

Hypercaloric diet study was originally designed to investigate non-alcoholic fatty liver disease and included 28 participants (170). For this study, plasma samples from 26 participants at the baseline and at the end of the study were available. The study was an

interventional, randomized, parallel-assigned study, where the participants ate 1000 extra calories per day for three weeks. The extra calories came predominantly from saturated fats (SAT group, n=13), unsaturated fats (UNSAT group, n=11) or simple sugars (CARB group, n=12).

4.1.7 Ethical statement

Written informed consent was obtained from all volunteers after the nature and potential risks of the study had been explained to them. All study protocols had been approved by local institutional review boards/ethics committees and the studies were conducted in accordance with the principles of the Declaration of Helsinki (172).

4.2 LABORATORY METHODS AND MATERIALS

The main materials and methods that were used in this study are described in **Table 3**. Detailed materials and methods can be found in studies I-III.

Table 3. The main materials and methods that were used to this study.

Method	*Reference*	*Study*
LDL isolation by ultracentrifugation	(173,174)	I, II, III
LDL modification	(82)	I, II, III
Dynamic light scattering	(83,175)	I, II, III
Lipid extraction	(176)	I, II, III
Mass spectrometry ESI-MS	(177-180)	I, II, III
LDL binding to proteoglycans	(181,182)	III
Circular dichroism	(83,183)	I
Cell culture	(93,184-186)	I
Mouse models	(187)	I
Statistical methods	-	I, II, III
ELISA and enzymatic assays	-	I

4.2.1 LDL isolation by ultracentrifugation

LDL (density = 1.019 to 1.063 g/ml) was isolated from frozen or fresh plasma/serum samples by KBr (potassium bromide) or D_2O (deuterium oxide) sequential ultracentrifugation (173,174). In Study I, KBr was used in the isolation of all of the samples and after standardizing the protocol for D_20, D_20 isolation was used (Study II and Study III). LDL concentrations for isolated LDL samples were expressed as protein concentrations measured by the bicinchoninic acid assay (BCA) protein assay (Pierce, Rockford, USA) or as the apoB-100 concentration as determined by ELISA (Mabtech, Nacka, Sweden). ApoB-100 measurements were done only for Health 2000 Health Examination Survey samples and

Sysdiet samples (Study I), after which the determination of the LDL protein with the BCA assay was found to correlate well with the amounts of apoB and BCA assay was used in all of the other samples.

4.2.2 LDL modifications

In the assay of LDL modification, KBr isolated plasma samples were first dialyzed extensively against a suitable buffer. Initially, when developing LDL aggregation susceptibility measurement, several agents were tested that have been shown to cause LDL aggregation (82). LDL oxidation was induced with 5 μM $CuSo_4$ in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Proteolysis of apoB-100 was induced with 0.1 mg/ml α-chymotrypsin from bovine pancreas (Sigma-Aldrich, Missouri, USA) in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 2 mM $CaCl_2$ and 2 mM $MgCl_2$. In the lipolysis of LDL, 50 mU/ml PLA_2 from bee venom (Sigma-Aldrich, Missouri, USA), 200 mU/ml neutral SMase from *Bacillus cereus* (bcSMase, Sigma-Aldrich, Missouri, USA), or 75 μg/ml human recombinant acid SMase (a kind gift from Genzyme, Study I or produced in house, Study II) were used. PLA_2 and bcSMase were incubated with LDL particles in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 2 mM $CaCl_2$ and 2 mM $MgCl_2$, and human recombinant SMase (hrSMase) in 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, pH 5.5, containing 150 mM NaCl and 50 μM $ZnCl_2$. PLA2 hydrolyses the fatty acid of PLs in the sn-2 position, resulting in the formation of FFA and LPC. SMase hydrolyses the phosphocholine head group of SM resulting in the production of ceramide and phosphocholine. SM is hydrolysed almost completely to ceramides within one minute, after which LDL starts to aggregate. In-house hrSMase was produced in collaboration with Associate Professor Olli Ritvos, University of Helsinki. (Study II and III)

4.2.3 Dynamic light scattering

The LDL particle size in native stage and LDL aggregates was detected with dynamic light scattering (DLS) (83,175). DLS is based on Brownian motion, defined as "the random movement of particles in a liquid due to the bombardment by the (solvent) molecules that surround them". Large particles have slow Brownian motions whereas smaller particles

move more quickly. In Study I, DLS Zetasizer Nano (Malvern Instruments, Malvern Works, UK) was used with cuvettes that were manually changed between measurements. The samples were incubated at 37°C between the measurements which were conducted once per hour for each sample for 6 hours. In studies II and III, the aggregation measurement was automatized and DLS Wyatt DynaPro Plate Reader II (Wyatt Technology, California, USA) was used in the measurements. In this method, samples were coated with paraffin oil to prevent sample evaporation and the data were collected with Dynamics V7 (Wyatt Technology, California, USA). The temperature was set to 37°C and each sample were measured every 15 to 30 minutes for 6 to 8 hours. The automatization of the method increased the sample size that was possible to measure per day by about 30-fold together with D_2O isolation (instead of KBr). The radius of native LDL particles starts to grow from native LDL with its 14 nm radius to heavily aggregated particles having a radius over 1000 nm. The accuracy of the dynamic light scattering measurement is limited to approximately 3000 nm because of the wavelength of the laser and thus the maximum size measured was limited to that size. From the raw data, time (h) – aggregate size (nm) curves are fitted using R-programme or GraphPad Prism and aggregate size at timepoint 2h or inflection point is determined.

4.2.4 Lipid extraction and mass spectrometry

Three different lipid mass spectrometers (MS)s were used to analyse the lipid composition of the samples. Lipidomics of LDL from the SYSDIET-study, the Ethnicity-study, the myriocin mice and the in vivo modified human LDL samples were analysed using Agilent 6490 or 6410 Triple Quad LC/MS with iFunnel technology (Agilent Technologies, Santa Clara, USA) in the Helsinki University Lipidomics Unit. Total lipids were extracted into chloroform-methanol (1:2, v/v) from blood plasma isolated LDL samples using Folch protocol (176). Prior to the analysis, lipid extracts were evaporated under a stream of nitrogen and dissolved in chloroform/methanol (1:2, v/v) and spiked with a mixture of quantitative internal standards. This standard included a separate standard for each analysed lipid class with a known concentration. A solution of 1% (v/v) NH_4OH or NH_3 was added to the samples just prior to the analysis to support ionization and prevent sodium adduct formation. The sample solutions were injected immediately via a syringe pump into the

electrospray ionization (ESI) source of a triple quadrupole mass spectrometer at a flow rate of 10 µl/min. Lipids with a choline head group (PC, LPC, SM) were detected by using an MS/MS precursor ion scan (precursors of m/z 184) and CE using MS/MS precursor scan of m/z 369. Triacylglycerols (TAG)s were analysed using an MS+ scan as $(M+NH_4)^+$ ions (177). The mass spectra data was analysed with Mass Hunter Workstation qualitative analysis software (Agilent Technologies, Inc.) and lipids were quantified using the internal standards and Lipid Mass Spectrum Analysis (LIMSA) software (188). The acyl chain assemblies were studied by recording their acyl chain either by recording negative ion mode product ion scans of the anion fragments for all common fatty acids (178). In this scan, formate adducts were used to serve as mother ions for choline lipids, which do not ionize in the negative mode as such, and the subsequent anionic fragments of the acyl chains were measured. In the other method, the acyl chain assemblies were studied by recording acyl chain specific MS/MS fragments (179). In this method, positive ion mode neutral loss scans of different acyl chains were detected for TAG species and negative ion mode precursor scans of the acyl fragments for PC species, of which formate adducts were used as mother ions.

Lipidomics for Corogene-study and Health 2000 Health Examination Survey were analysed using a 5500 QTRAP (SCIEX, Framingham, MA) mass spectrometer equipped with Eksigent 100-XL UHPLC system at Zora Biosciences (Espoo, Finland). Internal standard was added to the samples as well as protein precipitation solvent and lipids were extracted with acetate/isopropanol (2:8, v/v). Immediately prior to the analysis, 10 mM $NH_4CH_3CO_2$ in acetonitrile/2-propanol (4:3), v/v) with 0.1% formic acid was added in sample solution (180).

Lipidomics in the overfed study were analysed by using the UHPLC-QTOFMS system, Agilent Technologies (Santa Clara, CA, USA) combining 1290 Infinity system and 6545 quadrupole time of flight mass spectrometer (QTOFMS), interfaced with a dual jet stream electrospray (dual ESI) ion source at Örebro University, Sweden where lipids were extracted by using a slightly modified Folch method (189).

4.2.5 LDL binding to proteoglycans

Human aortas were obtained from autopsy within 24 hours of accidental death from the Department of Forensic Medicine, University of Helsinki (permission from the National Authority for Medicolegal Affairs; no 923/32/200/05). Proteoglycans were extracted from the intima media layer of the aortas as described before (181,182). 96-well plates were coated with intimal proteoglycans, blocked with 1 % BSA and 1µl of plasma was added and incubated at 37°C for 1 hour. Control wells were blocked with BSA, but not coated with proteoglycans. After washing unbound LDL and other lipoproteins from plates, the amount of total cholesterol was measured with the Amplex Red cholesterol assay kit (Molecular probes® by life technologies, Oregon, USA) by following the manufacturer's instructions.

4.2.6 Circular dichroism

Circular dichroism (CD) spectroscopy was performed as described before when analysing the apoB-100 conformation (83,183). LDL samples for CD analyses were diluted to 1 mg/ml in MES buffer, pH 5.5. The LDL samples were treated with SMase for 30 minutes and 10 mM EDTA was used to stop lipolysis. Control samples were treated identically, but without lipolysis. Lipolyzed and control LDL particles were diluted to 50 µg/ml for CD analysis and placed in 0.1 cm quartz cuvettes. CD spectra were registered with JASCO J-715 spectropolarimeter (Japan Spectroscopic Co.; Tokyo, Japan) in the region of 190-250 nm with step size 0.5 nm, scan speed of 50 nm/min, band width of 1 nm, and 1 s response at 37 ± 1 °C. Five spectra were obtained for each sample and average spectra were calculated from these after the blank measurements were subtracted.

CD signals at 222 nm, were used in the kinetic analysis of apolipoprotein folding. Molar ellipticity ([Θ]) at 222nm was calculated from the equation: $[\Theta] = (MRW) * \Theta / 10lc$, where Θ is a measured ellipticity in degrees, l is the cuvette path length (0.1 cm), c is the protein concentration (g/ml), and *the mean residue weight (MRW)* is obtained from the molecular weight and the number of amino acids. The α-helix contents were calculated from the equation using [Θ] at 222 nm: percent α-helix = [(-[Θ] 222+ 3,000)/(36,000 + 3,000)] * 100 (190,191).

4.2.7 Cell culture

Human primary macrophages

Human white blood cell fractions (buffy coats) were obtained from heathy volunteers (Finnish Red Cross Blood Service, Helsinki, Finland, (permission; no 1/2015 and 11/2016). Monocytes were plated to 8-well or 24-well plates (1.5 million cells per well), and the cells were allowed to attach for 1 h, washed three times with phosphate buffered saline (PBS) and serum free macrophage medium (GIBCO, Theromo Fisher Scientific, Co Dublin, Ireland) with 50 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF). The medium was changed every other day for one week, during which monocytes had differentiated into macrophages (93,184). Native LDL samples and LDL samples were pretreated with SMase for 4 or 24 hours and lipolysis was stopped with 10 mM EDTA; EDTA was added in equal amounts to the native LDL samples. Native LDL, SMase-aggregated LDL or acetylated LDL were added to macrophages in 24 well plates in serum-free RPMI 1640 media (Lonza, Walkersville, USA) containing 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. After an overnight incubation, the media was collected for matrix metalloprotease (MMP)-1, -2, -3, -7, -9, -10, -12, and -13 analyses. The concentrations of MMPs in culture media were measured using Human MMP Luminex Performance magnetic panel (R&D systems, Minneapolis, USA) according to the manufacturer's instructions. Cells were washed with PBS and intracellular lipids were extracted with hexane/isopropanol (3:2, v/v), 30 µl of lipid extract was pipetted into 96-wells, evaporated, and total cholesterol was determined with the cholesterol Amplex Red cholesterol kit (Molecular probes® by life technologies, Oregon, USA) according to the manufacturer's instructions.

To visualize the cholesterol accumulation in macrophage foam cells onto 13 mm glass coverslips in 8-well plates were washed after the incubation and fixed with 3.7 % formalin for 3 minutes at room temperature. Neutral lipids were stained with Oil Red O and counterstained with hematoxylin. Aquamount (BDH Laboratory Supplies, Poole, England) was used to mount coverslips on glass microscope slides and photographed (185).

T cells

48-5 T cell hybridomas from human apoB-100 transgenic mice were originally immunized against human oxLDL, but surprisingly responded only to native LDL, not to oxLDL (186). Irradiated (25 Gy) splenocytes were used as antigen presenting cells. C57BL/6J mice (Jackson, Bar Harbor, Maine) were sacrificed and spleens were harvested and meshed through a 100 μm cell strainer followed by osmotic lysis of red blood cells (EL buffer, Qiagen, Hilden, Germany). These mice were housed and treated in Karolinska Institutet, Stockholm, Sweden and study protocols were approved by the Stockholm North Committee for Experimental Animal Ethics. Splenocytes were co-cultured with 48-5 T-cell hybridomas and incubated with 10 μg/ml of native LDL or SMase aggregated LDL, or Concanavalin A as a positive control. The cells were incubated in 96-well plates with 200 μl serum-free RPMI 1640 medium containing ITS Premix (Corning, Bedford, Massachusetts), 0.1% bovine serum albumin, nonessential amino acids, L-glutamine, 1 mM sodium pyruvate, and 50 μM β-ME for 24 hours at 37°C in a humid 5% CO2 atmosphere. T cell activation was measured via the IL-2 concentration in the supernatant using ELISA (Mabtech, Nacka Strand, Sweden) according to the manufacturer's protocol.

4.2.8 Mouse models

Large ´empty´ vesicle-treated mice

Human *APOB*^{100} transgenic, *Ldlr*$^{-/-}$ -mice were housed in the University of Gothenburg, Sweden. Mice were on chow diet and food and water were served *ad libitum*. Large ´empty´ vesicles (LEVs, also known as unilamellar vesicles), having diameter of 100 nm were injected intravenously into mice once, with PBS being administered to the control mice. Mice were 13-19 weeks old and LEVs were dosed at 1000 mg PC/kg (body weight). One hour after injection of LEVs (n=16) or PBS (n=16), blood was collected, and plasma was isolated. LDL and VLDL fraction were isolated by KBr ultracentrifugation, but because LEVs can appear in LDL density range, VLDL-LDL samples were further purified. The LDL fraction was loaded into a Superose 6 HR column (size-exclusion FPLC), and eluted with 150mM NaCl, 1mM EDTA, pH 7.4. The first peaks for LEVs and VLDL were discarded, the subsequent LDL fractions were collected and pooled in pairs and re-loaded

onto the Superose 6 HR column, eluted with 150 mM NaCl, 1 mM EDTA pH 7.4; after this procedure, only LDL was detected. The animal procedures were approved by the Animal Ethical Committee at the Gothenburg University.

Myriocin treated mice

Ldlr$^{-/-}$, Apob$^{100/100}$-mice were housed in the National Laboratory Animal Center of the University of Eastern Finland. All mice were male and on chow diet until treatment with myriocin (n=11), or PBS (n=11) at the age of 14-22 weeks, when the animals were fed a high-fat atherogenic diet (21% milk fat, 0.2% cholesterol; Harlan Teklad; TD88137; also called the 'Western' diet), food and water were available *ad libitum.* Mice were injected intraperitoneally three times every week (Monday, Wednesday, Friday) for 10 weeks with myriocin (0.3mg/kg, Biomol Research Laboratories Inc.) or PBS. After the 10-week treatment, mice were fasted for 4h and terminal anaesthetised with isoflurane inhalation (Vetflurane 1000 mg/g, Virbab animal health), and blood was collected with heart puncture under deep anaesthesia. The aortas were collected and fixed with 4% paraformaldehyde (PFA) in PBS. These animal procedures were approved by the National Animal Experiment Board of Finland and carried out in accordance with the guidelines of The Finnish Act on Animal Experimentation.

Soat2$^{-/-}$ *mice*

Human *APOB*^{100} transgenic, *Ldlr*$^{-/-}$, *Soat2*$^{-/-}$ (n=5), and human *APOB*^{100} transgenic, *Ldlr*$^{-/-}$, *Soat*$^{+/+}$ mice (n=3) were housed in Wake Forest University. These mice are deficient in sterol O-acyltransferase 2 (SOAT2), an enzyme esterifying cholesterol; the animals are characterized by the enrichment of polyunsaturated CEs and TGs (187). Mice were fed for 8 months with a diet rich in cis-monounsaturated fat as described before (187). Plasma was collected and LDL particles were isolated with KBr ultracentrifugation. These animal procedures were approved by the Institutional Animal Care and Use Committee at Wake Forest University Health Sciences.

4.2.9 Statistical methods

The results are presented for normally distributed data as average ± SD (or SEM) or if data was not normally distributed as median and interquartile ranges. For normally distributed data, the statistical significance between groups was determined using Student´s t-test when comparing two groups and paired t-test when differences were tested within individuals of paired samples. One-way ANOVA was used in the comparison of more than two groups followed by Dunn´s post hoc tests. Mann-Whitney U or Kruskal-Wallis test was used to compare non-normally distributed data. A two-tailed Spearman correlation coefficient was used to analyse correlations between variables. If required, the test was followed by a post-hoc test. P-values ≤ 0.05 were considered significant. All statistical analyses were performed using IBM SPSS Software version 22.0, 23.0, 24.0 or 25.0 (IBM, North Castle, NY) or GraphPad Prism Software version 8.0.1 (La Jolla, CA).

5 RESULTS

5.1 LDL AGGREGATION IN ATHEROGENESIS

LDL aggregation in arterial intima is one of the crucial first steps in atherogenesis. Aggregated LDL particles cannot diffuse back to the circulation, they bind to intimal proteoglycans, resulting in LDL retention in the intima, and therefore they are prone to undergo further modifications (192). Macrophages phagocytose aggregated LDL and form foam cells, leading to plaque formation (89). In addition to other inflammatory cells, CD4+ T-cells, also called T helper cells, are present in atherosclerotic lesions and they have been demonstrated to be pro-atherogenic (97).

In addition to these previously known atherogenic properties of LDL aggregation, this thesis revealed that cultured human primary monocyte-derived macrophages, exposed to SMase-induced aggregated LDL led to massive foam cell formation and that these foam cells started to secrete increased amounts of MMP7 into the culture media (Study I, Figure 6A-C). In previous studies, MMP7 has been associated with plaque instability and adverse events in patients with carotid atherosclerosis (193). SMase-aggregated LDL also activated CD4+ T cells in an aggregate size dependent manner (Study I, Figure 6D). T cell activation was measured via IL-2 secretion and this was found to be blunted in the presence of oxLDL as shown before (186). CD4+ T cells are pro-atherogenic; they increase the level of inflammation and promote the development of a pathological lesion (194).

5.2 MEASUREMENT OF LDL AGGREGATION SUSCEPTIBILITY

One aspect of this thesis was the development of a method to measure LDL aggregation susceptibility from plasma or serum samples. In the devised method, LDL was isolated from blood by sequential ultracentrifugation, then LDL aggregation was induced by incubation with hrSMase with the LDL particle size being followed with dynamic light scattering. The LDL aggregate size at timepoint 2-h clearly correlated with the inflection point and could

pinpoint individuals with aggregation prone LDL (Study I, Supplementary Figure 3A-C, Study II, Figure 1B-C).

It was found that LDL aggregation varied between individuals, and furthermore each of tested LDL aggregation methods i.e. oxidation, proteolysis, and lipolysis by PLA_2, bcSMase and hrSMase all revealed a similar separation between individuals. Of these methods, hrSMase resulted in the formation of the largest sized aggregates, and the greatest differences between individuals and therefore, it was chosen for systematic analysis of the samples. Importantly, LDL aggregation susceptibility did not correlate with common risk factors for ASCVD, such as age, smoking, gender, LDL-C, apoB-100, Lp(a) or high sensitivity C-reactive protein (hsCRP). (Study I, Supplementary Tables 1 and 2).

The LDL concentration at the beginning of the incubation with hrSMase was found to influence the LDL aggregation, i.e. the higher the concentration, the faster the aggregation. A concentration of 0.2 mg/ml was chosen for the standard protocol, because LDL aggregation changed only relatively marginally if the LDL concentration was increased or decreased from the 0.2 mg/ml (Study I, Supplementary Figure 2A).

The inter – assay variance was found to be small in an experiment where the plasma from a single individual was divided into 26 aliquots and separately isolated and analyzed. Plasma freezing to -80 °C once, did not affect LDL aggregation and freezing – thawing up to five times had only a small effect with this difference only being detectable after 4-h incubation. Intra-individual day-to-day variance was found to be minor (Study I, Supplementary Figure 2B-D).

5.3 LDL LIPID COMPOSITION ALTERS THE APOB-100 CONFORMATION AND THEREBY CHANGES THE LDL AGGREGATION SUSCEPTIBILITY

To examine the associations between LDL susceptibility and LDL lipid composition, both were analysed from the same samples from 100 healthy individuals (Health 2000). The LDL lipid composition was determined using quantitative lipid mass spectrometry based

lipidomics. It was discovered that LDL aggregation susceptibility was positively associated with several SM and Cer species and negatively associated with several PC species (Study I, Figure 2B). With respect to the core lipids, there were no clear associations with LDL aggregation susceptibility. However, 48-52 carbon TAGs correlated negatively with the LDL aggregation susceptibility in Health 2000 study (Study I, Figure 3A). Furthermore, the lipid-LDL aggregation susceptibility association was consistently found in all of the following cohorts examined in this thesis i.e. Corogene (Study I, Figure 2B,3B), SYSDIET (Study I, Figure 4C, changes), Equator (Study I, Figure 4F, changes), Ethnicity (Study II, Figure 2A) and Hypercaloric (Study III, Supplemental figure 4A,B) studies.

The causality of surface lipid composition to LDL aggregation susceptibility was studied *in vivo* and *in vitro*. Isolated LDL particles were directly enriched *in vitro* with SM, PC or LPC and LDL lipid composition, LDL aggregation susceptibility and apoB-100 conformation were evaluated from the original LDL samples and the lipid-enriched LDL samples. The changes in relative proportions of SM, PC and LPC were in a similar range as had been observed between individuals (Study I, Supplementary Figure 4A). The LDL particles enriched in SM became more prone to aggregation, whereas particles enriched in PC or LPC became more resistant against aggregation (Study I, Supplementary Figure 4B). It had been shown earlier that SMase treatment of LDL particles induced changes of apoB-100 conformation by decreasing the α-helical structures of apoB-100 (83). One mechanistic explanation for this finding is that the changes in the surface lipid composition altered apoB-100 conformation already in non-lipolyzed LDL particles. Furthermore, SM enrichment enhanced SMase-induced conformational changes of apoB-100 (Study I, Supplementary Figure 4C, D).

For the in *vivo* experiments, we chose mouse models that had been previously shown to change LDL lipid composition and reduce atherosclerosis without affecting the plasma LDL concentration (195-197). The purpose of the first mouse model was to increase the amount of LDL-PC. Human *APOB100 transgenic/Ldlr*$^{-/-}$ mice were intravenously injected with a single dose of large empty vesicles (n=16), composed of PC 16:0/18:1; the control group of *APOB100 transgenic/Ldlr*$^{-/-}$ littermates, received equivalent dose of PBS (n=16). Both SM/PC and UC/CE were decreased in LEV treated mice group compared to controls, the

SM/PC ratio was three-fold lower and the UC/CE ratio was two-fold lower. LEV treatment led to the formation of almost completely aggregation-resistant LDL i.e. even after overnight incubation with hrSMase, the LDL aggregate size was found to be small (Study I, Figure 5A).

The purpose of second mouse model was to reduce LDL-SM by suppressing SM biosynthesis. *Ldlr*$^{-/-}$*/Apob*$^{100/100}$ mice were injected intraperitoneally with either myriocin or PBS three times per week for ten weeks, simultaneously while consuming a high fat diet (n=22). Myriocin is an inhibitor of serine-palmitoyl transferase, the first step and the rate-limiting enzyme in the biosynthesis of SM. Plasma levels of cholesterol and TGs increased in both groups equally due to high fat diet, but the SM/PC ratio was halved in the myriocin treated mice group. (Study I, Supplementary Figure 8) With this model, we also could demonstrate that atherosclerosis was decreased in the myriocin treated group (Study I, Figure 5C), as shown previously in a different atherogenic mouse model (196). Finally, LDL aggregation was decreased in myriocin treated mice group when compared to control mice (Study I, Figure 5B).

In the third mouse model, it was investigated if modification of core lipids would exert an effect on the LDL aggregation susceptibility. For this purpose, SOAT2 deficient mice were used; these mice lack the enzyme known alternatively as sterol-O-acyltransferase 2, or cholesterol acyl-CoA acyltransferase-2 (ACAT2). SOAT2 esterifies cholesterol predominantly in the liver and small intestine (198,199), and previously inhibition of SOAT2 has been shown to reduce atherosclerosis (200). *Soat2*$^{-/-}$ mice are characterized by the enrichment of polyunsaturated CE and TAG in LDL particles in their blood as compared to *Soat2*$^{+/+}$ littermates (187). In this study, LDL was isolated from blood samples of *APOB100 transgenic/Ldlr*$^{-/-}$*/Soat*$^{-/-}$ mice (n=5) and *APOB100 transgenic/Ldlr*$^{-/-}$*/ Soat2*$^{+/+}$ littermates (n=3). The LDL particles in the blood of SOAT2 deficient mice was found to be extremely resistant to aggregation (Study I, Figure 5D).

5.4 AGGREGATION PRONE LDL PREDICTS FUTURE ASCVD DEATHS INDEPENDENTLY OF CONVENTIONAL RISK FACTORS

Plasma LDL-C is an independent, causal risk factor for ASCVD and LDL aggregation is one of the first steps in atherogenesis (4,201). However, conventional risk factors, including plasma LDL-C do not identify all patients at risk for MI. The amount of patients with zero conventional risk factors who nonetheless suffer an MI varies from 8 to 41%; this value, is higher in males, and increases with age (202). The majority of patients that experience out-of-hospital sudden cardiac arrest have no symptoms prior the event or their risk have been classified as low risk if disease is previously identified (203,204). Clearly new risk factors are needed to identify and then to treat these patients, who carry this insidious and unrecognised risk for MI. Here, it was studied if LDL aggregation susceptibility would associate with CADs and/or CAD death independently of conventional risk factors.

In these experiments, samples were analyzed from a nested case-control study (31), which was originated from the prospective Finnish Corogene study (168). Patients were assigned for coronary angiogram and patients with at least 50% stenosis in their coronary arteries were chosen for this study. Exclusion criteria for the substudy included diabetes, heart transplantation, previous blood transfusion and low haemoglobin and female sex. Blood samples were taken and then patients were followed for an average of 2.5 years. Those patients that suffered a coronary death within the follow-up period were designated as the case group (CAD Death group, n=24). Controls were chosen from the group of patients that experienced no cardiovascular events during the follow-up time (Stable CAD group, n=24) by pairwise matching them with cases based on conventional risk factors, coronary stenosis index and statin use. Inter-individual variation in LDL aggregation susceptibility was observed as described earlier among healthy subjects. Importantly, LDL aggregates were significantly larger at the 2 h time point in the CAD Death group (median 1500 nm) when compared to the Stable CAD group (median 940 nm, $p<0.05$). Both groups, with diagnosed CAD, were observed to have very significantly larger LDL aggregate sizes compared to

LDL aggregates from healthy individuals from Health 2000 study participants (median 200 nm, $p<0.001$ for both) (Study I, Figure 1D). LDL aggregation susceptibility, measured as a particle size at 2 h, did not correlate with plasma LDL-C, apoB-100, HDL-C, total cholesterol, Lp(a) or high sensitivity hsCRP, nor with age, BMI, statin use, smoking or LDL particle size. (Study I, Supplementary Table 2) In Corogene study and in Health 2000, a negative correlation with TGs was observed with the LDL aggregation susceptibility (Study I, Supplementary Tables 1 and 2).

5.5 LDL OF SOUTH ASIANS IS MORE PRONE TO AGGREGATION COMPARED TO WHITE CAUCASIANS

South Asians have a higher risk to develop ASCVD in comparison to other ethnic groups (40,41). To some extent, this is due to their higher prevalence of traditional ASCVD risk factors, but it is not fully explained by these factors (42-44). It was examined if LDL aggregation is higher in healthy young South Asians (n=12, age 27.5 ± 3.2 years) compared to white Caucasian males (n=12, age 25.6 ± 3.2 years). All the study subjects lived and had been born in the Netherlands, and the groups were matched according to their age, BMI, blood pressure, plasma TG, LDL-C, HDL-C, insulin and glucose. I observed, similarly to others (205,206), that South Asians have a higher body fat % than white Caucasians (19 % vs. 15 %, $p=0.015$) with a similar BMI. (Study II, Table 1) The LDL aggregation susceptibility was observed to be higher within South Asians compared to white Caucasians (aggregate size at 2h; 620 ± 320 nm vs 350 ± 290 nm, $p = 0.011$). (Study II, Figure 1D). The LDL aggregation susceptibility positively correlated with body fat % (Study II, Figure 4A), and both LDL aggregation susceptibility and body fat % correlated positively with SM 24:0 and TAG 56:8 and negatively with CE 18:1 in South Asians (Study II, Figure 2 and 4).

5.6 LDL AGGREGATION SUSCEPTIBILITY IS MODIFIABLE WITH DIET

A Healthy Nordic diet has been shown to be beneficial for cardiovascular health (161,162). Here, the effect of diet for LDL aggregation was studied in three different intervention. In SYSDIET –study (167,207), where participants were randomized to eat Healthy Nordic diet or control diet. All study subjects were overweight and had in addition two features of the metabolic syndrome, but without type 2 diabetes. In the healthy Nordic diet group, the participants were instructed to consume whole grain products, an abundance of fruits and vegetables and fatty fish among other recommendations. In the control group, the diet was designed based on the mean consumption of nutrients in the Nordic countries and for example, the amount of fibres, and fruits and vegetables was limited for this reason. Key foods were provided for both groups. The diets were isocaloric and lasted for 18 to 24 weeks. Four-day food diaries were collected at the baseline and at the end point. The clinical characteristics and the macro- and micro-nutrient amounts of the study subjects are presented in Study I, Supplementary Table 3 and Supplementary Figure 5A.

It was studied here if LDL aggregation susceptibility would change from samples taken at the baseline and at the end of the intervention in both groups. It was found that the level of LDL aggregation decreased in two third of the cases in individuals in the Healthy Nordic diet group, but increased in one third of the cases (Study I, Figure 4A). In the control group, only small changes were observed in LDL aggregation susceptibility (Study I, Figure 4B). In the Healthy Nordic diet group, changes in the LDL aggregation susceptibility correlated positively with LDL-SMs, and negatively with LDL-PCs (Study I, Figure 4C). It was analysed further if these changes in LDL lipids and LDL aggregation could be explained by changes in diet in the Healthy Nordic diet group. The multivariate model identified two factors that best explained the changes in LDL aggregation susceptibility, changes in dietary vitamin E and changes in dietary sucrose (Study I, Table 4). The increase in dietary vitamin E was associated with a decrease in LDL aggregation susceptibility, whereas an increase in dietary sucrose was associated with increased LDL aggregation susceptibility. Dietary vitamin E was a marker of the intake of polyunsaturated fatty acid rich vegetable oils, not a

supplement. Dietary vitamin E and dietary polyunsaturated fatty acids were associated positively with LDL-PC and negatively with LDL-SM, and were considered to be strongest beneficial factors modifying both the LDL lipid composition and LDL aggregation susceptibility (Study I).

Next, it was studied in an extreme study setting if over-consumption of saturated fats, unsaturated fats of simple sugars would exert any effect on LDL lipids and LDL aggregation susceptibility. All the study subjects were overweight also in this study, and randomized to consume 1000 extra calories per day in addition to their regular diets for three weeks. The clinical characteristics of the study subject were described in Study III, Table 2. The extra calories came predominantly from saturated fats (SAT group), unsaturated fats (UNSAT group) or simple sugars (CARB group). The extra food was provided for each group, and they consisted of blue cheese, butter and coconut oil in SAT group, olive oil, pesto, pecan nuts and a small amount of butter in the UNSAT group, and candy, sugar-sweetened beverage and orange juice in CARB group (Study III, Table 1). Fasted plasma samples were collected at the baseline and after the intervention. The compliance of the diets was verified by analysing VLDL-TGs; in the CARB group, the amounts of unsaturated fatty acid-TAGs were increased due to *de novo* lipogenesis, producing mainly palmitic acid (16:0). In the SAT group, saturated-TAGs were increased and unsaturated-TAGs decreased, while in UNSAT group unsaturated TAGs increased (Study III, Figure 1).

It was studied if any of the diets had any effect on the atherogenic properties of LDL i.e. LDL aggregation susceptibility, LDL lipidomics, LDL binding to human proteoglycans or LDL oxidation. In the CARB group, it was found that the amount of saturated-TAGs in LDL particles was increased and that of unsaturated-TAGs had decreased, in line with VLDL lipid analyses. In addition, there were small, inconsistent changes in LDL-PC species (Study III, Figure 4C). The CARB diet had no effect on any of the measured functional properties of LDL, LDL aggregation (Study III, Figure 5C), LDL binding to proteoglycans (Study III, Figure 2B, C) or LDL oxidation (Study III, Figure 3B, C). In the UNSAT group, many LDL-PC species were slightly, but consistently decreased, total TAGs were decreased, and CEs increased (Study II, Figure 4A). The level of LDL binding to human proteoglycans was significantly decreased in the UNSAT group but the diet had no effect on other functional

properties of LDL particles (Study III, Figures 2B, C, 3B, C, 5C). In the SAT group, most substantial changes in LDL lipids were found, most importantly many LDL-SMs were increased. In addition, the amounts of saturated fatty acids containing PCs and TAGs were increased and unsaturated fatty acids containing PCs and TAGs were decreased (Study III, Figure 4B). Most importantly, the LDL aggregation susceptibility was increased in the SAT group (Study III, Figure 5C), and this increase associated positively with LDL-SM species and negatively with LDL-PC species (Study III, Figure 5E).

5.7 LDL AGGREGATION SUSCEPTIBILITY IS DECREASED WITH PCSK9 MONOCLONAL ANTIBODY TREATMENT

PCSK9 inhibition with mAbs dramatically lowered the amount of LDL-C and this is recommended for patients at a high risk for ASCVD events. The LDL aggregation susceptibility did not correlate with the LDL-C concentration in any of these studies (I, II, III), however we have found that PCSK9 mAb treatment altered both the plasma and lipoprotein PL compositions (208).

The samples were obtained from a randomized, double-blind, phase 2 study of fully human immunoglobulin G1 (IgG1) mAb against PCSK9 (RG7652), called the EQUATOR study. RG7652 (n = 25) or placebo (n = 15) were once injected subcutaneously to the patients, and blood samples were taken before and 29 days after the treatment. The LDL aggregation susceptibility was decreased in two out of every three RG7652-dosed patients, in the placebo group, LDL aggregation slightly decreased in one out of every three samples (Study I, Figure 4D-E). Changes in LDL aggregation susceptibility were significantly different ($p = 0.035$) between the groups. In the RG7652-dosed group, changes in LDL aggregation susceptibility correlated negatively with total PC and several polyunsaturated PC species, and positively correlated with SM 18:1/16:1 and SM 18:1/22:0 (Study I, Figure 4F).

6 DISCUSSION

Despite the administration of effective LDL-C lowering therapies, the risk for ASCVD remains and as many as every second person dying from a CAD event does not experience any symptoms prior to the event (12). Therefore, much research still needs to be done in order to detect and treat these patients who carry some unrecognized risk(s) for CAD. This study focused on examining LDL aggregation susceptibility from a mechanistic viewpoint and as a potential predictive risk-factor for CAD death.

Even though the role of LDL aggregation in atherogenesis is well established (36,82,84-90,209), it has not been proposed before as a measurable risk factor for ASCVD. A high LDL concentration is itself a risk factor for ASCVD and LDL is more likely to aggregate when present at higher concentrations. Therefore lowering the LDL concentration is one way to reduce the tendency of LDL aggregation in the artery wall. This thesis shows that there is extensive, inter-individual variation in LDL aggregation susceptibility (Study I), and those individuals who have the aggregation prone LDLs might benefit most from intensive LDL-C lowering therapies. First and foremost, this thesis showed that LDL aggregation susceptibility, measured from plasma samples, predicted future CAD deaths in a group of patients with established coronary atherosclerosis, independently of conventional CAD risk factors including plasma LDL-C concentration, smoking, and hypertension (Study I). (**Figure 8**) Because of the relatively small number of examined patients, the present results will need to be verified with a larger cohort, and also in geographically different populations, and different patient groups. The preliminary data from a study, where patients with severe PAD undergoing lower revascularization, indicates that LDL aggregation is increased among PAD patients (n=239) in comparison to sex, age and ethnicity matched controls (n=20, $p<0.001$) (210). In addition, pre-operative LDL aggregation predicted post-operative major cardiovascular events ($p=0.02$) when corrected for conventional risk-factors (210).

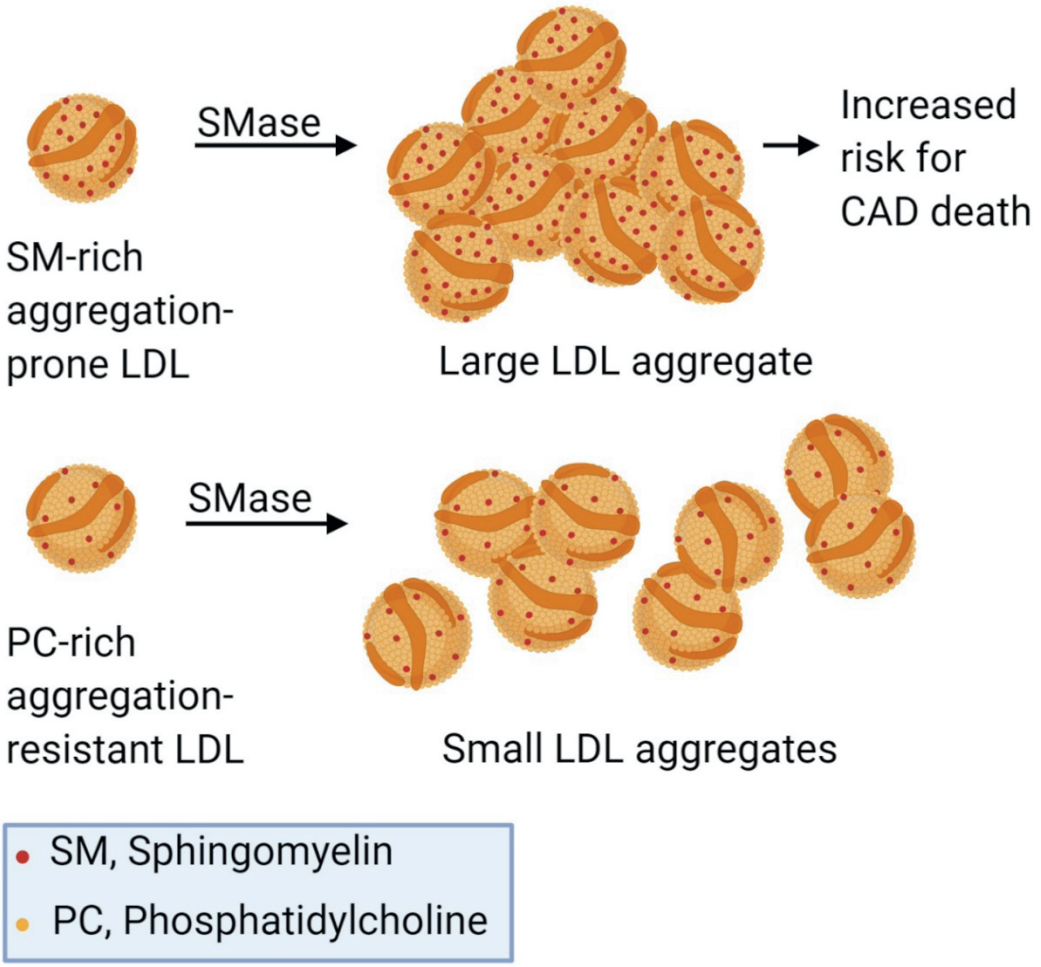

Figure 8. LDL aggregation. Sphingomyelin-rich LDL particles isolated from plasma samples are prone to aggregate and the presence of aggregation-prone LDL particles predicts future CAD deaths independently of conventional CAD risk factors. LDL particles that are more resistant to aggregation are enriched with phosphatidylcholines. SMase, Sphingomyelinase, CAD, coronary artery disease. Figure created with BioRender.com.

In order to mechanistically explain inter-individual variation in LDL aggregation susceptibility, a detailed lipidomic analysis was performed in seven different cohorts. The aggregation prone LDL particles consistently associated with a higher relative proportion of SMs and aggregation resistant LDL particles associated with a higher relative proportion of

PCs (Studies I-III). Plasma sphingolipids, which are carried in plasma in lipoprotein particles, have been related to increased atherosclerosis and cardiovascular deaths (31-34). Our results may partly explain the mechanism behind these findings. Further associations between plasma sphingolipids and LDL aggregation susceptibility remain to be clarified.

Form the clinical viewpoint, it is relevant to determine whether LDL aggregation susceptibility can be modified. To study if this was the case, the LDL composition was pharmacologically and genetically modified *in vivo*. Increasing LDL-PC by vesicle treatment, decreasing LDL-SM by inhibiting SM biosynthesis and increasing polyunsaturated TAGs and CEs, all resulted in a reduced LDL aggregation susceptibility (Study I). Similarly, direct *in vitro* enrichment of isolated LDL particles with SM increased LDL aggregation and PC or LPC enrichment decreased LDL aggregation. These *in vivo* and *in vitro* interventions verified the causal role of LDL lipids in the LDL aggregation susceptibility but were not relevant clinical approaches. Therefore, it was further studied if diet, genetic background or medication would be capable of affecting an individual's LDL aggregation susceptibility (Studies I-III).

First, the Healthy Nordic diet study showed that vegetable oil consumption, measured as dietary vitamin E intake, was associated with reduced LDL aggregation, while the consumption of sugars best explained the elevated LDL aggregation (Study I). In an extreme study setting, it was further investigated if overconsumption of saturated fats, unsaturated fats, or simple sugars would alter the LDL lipid composition and the LDL aggregation tendency. It was observed that overconsumption of saturated fats did result in increased levels of LDL-SM and increased LDL aggregation, whereas unsaturated fats or sugars did not have any effect on LDL aggregation (Study III). The increase in the level of LDL-SM is likely due the fact that a saturated fat diet is rich in palmitate, a precursor of palmitoyl-CoA, a rate-limiting substrate of SM biosynthesis (211,212). Dietary saturated fats are also known to increase plasma LDL-C levels, and CAD risk (4,128). Our results here support the harmfulness of dietary saturated fats for cardiovascular health.

Overconsumption of unsaturated fats decreased both the plasma oxLDL concentration and LDL binding to human aortic proteoglycans, the other proatherogenic characteristics of LDL

(Study III). Vegetable oil supplementation (rich in linoleic or oleic acid) has been shown also to reduce LDL binding to proteoglycans by our research group (213) and others (214). Dietary unsaturated fat, despite not affecting LDL aggregation susceptibility ((Study III) and (213)), does have other beneficial effects with regard to the LDL quality by reducing oxLDL and LDL binding to proteoglycans. However, the most beneficial effect on plasma lipids has been obtained when replacing dietary saturated fats with *cis*-PUFA (predominantly linoleic acid and α-linolenic acid) (128). Accordingly, in the future it would be interesting to study whether this kind of intervention exerts a beneficial effect also on the LDL aggregation susceptibility. I have also shown that consumption of *Camelina sativa* oil, which is rich in linolenic acid (18:3), does not affect LDL SM/PC ratios or LDL aggregation (213). In the line with UNSAT group, LDL binding to human proteoglycans was decreased by treatment with *Camelina sativa* oil (213), but this decrease seemed to be dependent on plasma LDL-C levels.

Replacing saturated fats with unsaturated fats, is only marginally effective in overweight and obese individuals (215,216). The participants in Healthy Nordic diet study (Study I), overconsumption study (Study III) and *Camelina sativa* oil study (213) were all obese. Obesity triggers adipose tissue induced inflammation that leads to insulin resistance (217,218) and insulin resistance is known to disturb lipid metabolism, and may lead to elevated cholesterol synthesis and lower cholesterol absorption (219,220). The response to dietary changes in obese people compared to normal weight people is somewhat limited for these reasons (216). It is very likely that diet- induced changes in LDL aggregation susceptibility are also limited in obese individuals, since LDL aggregation susceptibility depends on LDL lipid composition. The plasma lipid composition of obese individuals is most likely to be affected by their excess adipose tissue. In non-human primates, it has been recently shown that the levels of several plasma SM species, as well as palmitoleic acid (C16:1) and arachidonic acid (C20:4) are higher among obese individuals (221). In addition, obese humans have more palmitoleic and arachidonic acid in their adipose tissue when compared to their non-obese counterparts (222).

Finally, it was studied if LDL aggregation susceptibility differed between two different genetic pools, South Asians and white Caucasians. LDL obtained from South Asians was

more prone to aggregate when compared to white Caucasians (Study II), and this may partly explain the higher risk of South Asians for CAD (40,41). It was observed here that LDL-SMs were associated with a higher body fat percentage, and conversely LDL-PCs associated with a lower body fat percentage in the whole population studied. Furthermore, the LDL aggregation susceptibility was found to positively correlate with body fat percentage. This, together with the previous findings of the thesis, suggest that overweight/obese individuals may have more aggregation prone LDL. Unfortunately, in these studies it was not possible to detect a correlation between BMI and LDL aggregation susceptibility because of the low variation in the BMI values. The genetic influence on LDL aggregation susceptibility remains to be studied in detail. For example, the genes which regulate the expression of enzymes synthesising SM or PC are potential candidates as are the genes coding for lipoprotein and lipid receptors.

Ingested plant stanol esters reduce LDL absorption from the intestine, resulting on average of a 10-15% reduction in the plasma LDL-C concentration (138,223). The effective dose is 2-3 g per day (138) which cannot be achieved by any means other than consumption of plant stanol ester enriched foods. The preliminary results suggest that the consumption of plant stanol enriched rapeseed oil-based spread (3 g of plant stanol esters per day) for 6 months significantly ($p=0.0010$) was capable of decreasing the LDL aggregation susceptibility (224).

LDL aggregation susceptibility does not associate with the LDL-C concentration but can be reduced by consumption of lipid lowering plant stanol esters. Obviously, this raises the question if lipid lowering medication also decreases LDL aggregation susceptibility. In this study, only PCSK9-mAb treatment, an extreme lipid lowering drug was studied; PCSK9-mAb treatment successfully reduced the LDL aggregation susceptibility. As statins are the most common lipid lowering drugs, it would be important to study how statins affect the LDL aggregation susceptibility. There is also the potential to develop new treatments aimed at enhancing the LDL quality on an individual basis.

From a pathological viewpoint, our results deepen the knowledge of how plasma LDL promotes atherogenesis in the artery wall. The higher the concentration of plasma LDL-C,

the greater the concentration of LDL which is present in arterial intima. At higher concentrations, LDL particles are more likely to aggregate and this can induce enhanced inflammatory responses (Study I). The observed elevated activation of T cells with SMase-aggregated LDL is most likely due to increased phagocytosis of LDL-aggregates of antigen presenting cells, and this can lead to a further increase in antigen presentation to the T cells.

In summary, this thesis describes a novel measurable and modifiable risk factor for CAD, i.e. LDL aggregation susceptibility. The future will reveal the potential of LDL aggregation susceptibility measurement in clinical use, for example, whether the determination of this parameter can help select those patients that will benefit the most from treatment with potent lipid lowering drugs.

7 CONCLUSIONS & FUTURE PERSPECTIVES

The conclusions by this work are presented in **Figure 9**.

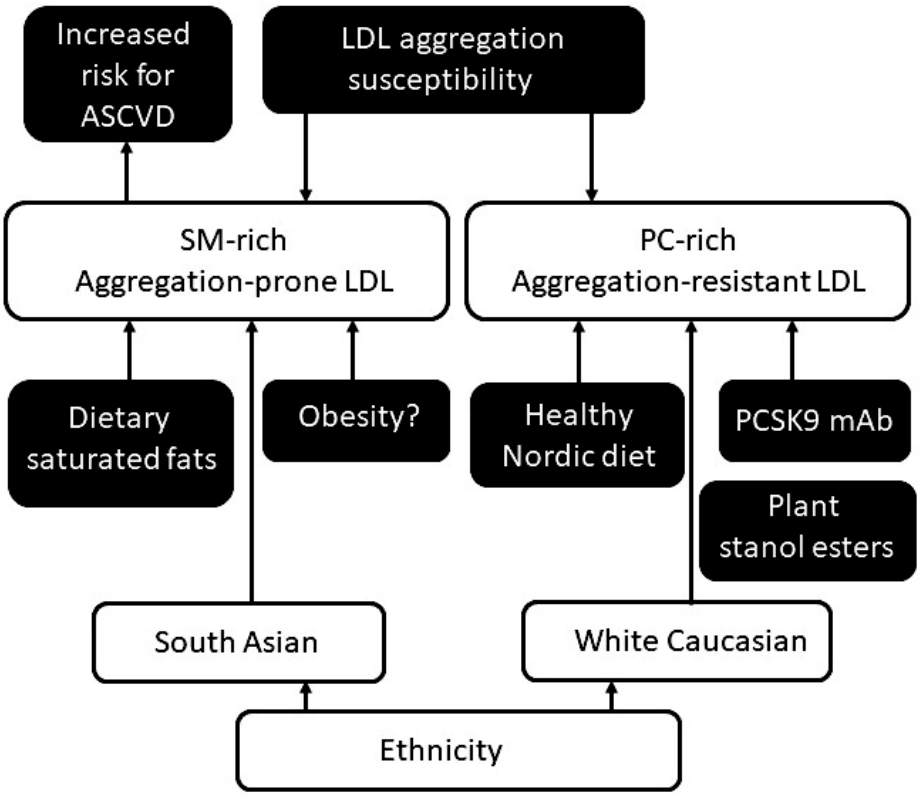

Figure 9. Conclusions of this Thesis. The LDL aggregation susceptibility measured from plasma/serum samples varies among individuals. The types of LDL particles that are prone to aggregate are enriched in sphingomyelins (SMs) whereas LDL particles that are enriched in phosphatidylcholines (PCs) are resistant to aggregation. Aggregation-prone LDL is associated with atherosclerotic cardiovascular disease (ASCVD) and predicts ACVD deaths. Dietary saturated fats increase LDL aggregation, and there is some evidence to suggest that LDL from obese individuals aggregate more than LDL from normal-weight individuals. LDL aggregation susceptibility can be reduced with dietary plant stanol ester supplementation, by adopting a Healthy Nordic diet and by PCSK9 monoclonal antibody (mAb) treatment. Ethnic background has an effect on LDL aggregation susceptibility, the LDL obtained from Dutch men of South Asian origins tends to aggregate more compared than that from white Caucasian Dutch men.

The main finding emerging from this thesis is that LDL aggregation is not simply a pathological event in artery wall leading to plaque formation, but it is a measurable risk factor of ASCVD. This thesis demonstrates that LDL aggregation susceptibility varies among individuals and can be modified. Dietary supplementation of plant stanol esters reduces LDL aggregation (224). In obese subjects, LDL aggregation susceptibility is decreased by changing to eating a Healthy Nordic diet whereas it is increased by overconsumption of saturated fats; it is not affected by unsaturated fats or sugars. The effect of different diets, dietary supplements and the effect of body composition need to be clarified in order to verify and characterize these findings in greater detail.

PCSK9 inhibition decreased the LDL aggregation susceptibility and may contribute to the preventative effect of PCSK9 mAb treatment in high risk individuals. Statin and ezetimibe treatment may also decrease LDL aggregation, but this will need to be examined. From a mechanistic point of view, the effects of the various lipid lowering drugs that have different mechanisms of action will provide more information about how LDL lipid composition and the aggregation susceptibility are altered.

LDL aggregation susceptibility was found to be different between different ethnic groups. LDL from young, healthy South Asians is more prone to aggregate when compared to matched white Caucasians. The genetics behind individual differences in LDL aggregation susceptibility has not been studied, but will potentially explain the majority of these differences, since the modifications achievable with diet, dietary supplements or medication were modest when compared to inter-individual differences.

Together with Katariina Öörni and Petri Kovanen, I have applied for a patent for the analysis of LDL aggregation susceptibility. The newly devised method improved and accelerated considerably these assays, but further developments to allow the high throughput isolation for LDL particles will likely be needed before this technique has widespread clinical applications.

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9 REFERENCES

1 Mohsen Naghavi, Amanuel Alemu Abajobir, Cristiana Abbafati, Kaja M Abbas, Foad Abd-Allah, Abera SF, et al. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet.* 2017; 390: 1151-210.

2 WHO. *World Health Organization. Fact sheets. Detail. Cardiovascular diseases (CVDs) [referred: 14.11.2019]*, <https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) > (2017).

3 Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, et al. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *Eur Heart J.* 2019; 00: 1-79.

4 Ference BA, Ginsberg HN, Graham I, Ray KK, Packard CJ, Bruckert E, et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur Heart J.* 2017; 38: 2459-72.

5 Yurdagul A, Jr., Finney AC, Woolard MD, Orr AW. The arterial microenvironment: the where and why of atherosclerosis. *Biochem J.* 2016; 473: 1281-95.

6 Adamson S, Leitinger N. Phenotypic modulation of macrophages in response to plaque lipids. *Curr Opin Lipidol.* 2011; 22: 335-42.

7 Myerburg RJ, Interian A, Jr., Mitrani RM, Kessler KM, Castellanos A. Frequency of sudden cardiac death and profiles of risk. *Am J Cardiol.* 1997; 80: 10F-9F.

8 Myerburg RJ, Kessler KM, Castellanos A. Sudden cardiac death: epidemiology, transient risk, and intervention assessment. *Ann Intern Med.* 1993; 119: 1187-97.

9 Chugh SS, Reinier K, Teodorescu C, Evanado A, Kehr E, Al Samara M, et al. Epidemiology of sudden cardiac death: clinical and research implications. *Prog Cardiovasc Dis.* 2008; 51: 213-28.

10 Huikuri HV, Castellanos A, Myerburg RJ. Sudden death due to cardiac arrhythmias. *N Engl J Med.* 2001; 345: 1473-82.

11 Zipes DP, Wellens HJ. Sudden cardiac death. *Circulation.* 1998; 98: 2334-51.

12 Katritsis DG, Gersh BJ, Camm AJ. A Clinical Perspective on Sudden Cardiac Death. *Arrhythm Electrophysiol Rev.* 2016; 5: 177-82.

13 Keele KD. Leonardo da Vinci's views on arteriosclerosis. *Med Hist.* 1973; 17: 304-8.

14 daVinci L. *I Manoscritti Di Leonardo Da Vinci Della Reale Biblioteca Di Windsor: Dell'anatomia, Fogli B, Pubblicati Da Teodoro Sabachnikoff.* (Roux e Viarengo Editori, 1901).

15 Thompson RC, Allam AH, Lombardi GP, Wann LS, Sutherland ML, Sutherland JD, et al. Atherosclerosis across 4000 years of human history: the Horus study of four ancient populations. *Lancet.* 2013; 381: 1211-22.

16 McNamara JJ, Molot MA, Stremple JF, Cutting RT. Coronary artery disease in combat casualties in Vietnam. *JAMA.* 1971; 216: 1185-7.

17 Enos WF, Holmes RH, Beyer J. Landmark article, July 18, 1953: Coronary disease among United States soldiers killed in action in Korea. Preliminary report. By William F. Enos, Robert H. Holmes and James Beyer. *JAMA.* 1986; 256: 2859-62.
18 Relationship of atherosclerosis in young men to serum lipoprotein cholesterol concentrations and smoking. A preliminary report from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. *JAMA.* 1990; 264: 3018-24.
19 Strong JP, Malcom GT, McMahan CA, Tracy RE, Newman WP, 3rd, Herderick EE, et al. Prevalence and extent of atherosclerosis in adolescents and young adults: implications for prevention from the Pathobiological Determinants of Atherosclerosis in Youth Study. *JAMA.* 1999; 281: 727-35.
20 Allison MA, Criqui MH, Wright CM. Patterns and risk factors for systemic calcified atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2004; 24: 331-6.
21 Lanier JB, Bury DC, Richardson SW. Diet and Physical Activity for Cardiovascular Disease Prevention. *Am Fam Physician.* 2016; 93: 919-24.
22 Smith EB. Transport, interactions and retention of plasma proteins in the intima: the barrier function of the internal elastic lamina. *Eur Heart J.* 1990; 11 Suppl E: 72-81.
23 Goldstein JL, Brown MS. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu Rev Biochem.* 1977; 46: 897-930.
24 Wiviott SD, Cannon CP, Morrow DA, Ray KK, Pfeffer MA, Braunwald E. Can low-density lipoprotein be too low? The safety and efficacy of achieving very low low-density lipoprotein with intensive statin therapy: a PROVE IT-TIMI 22 substudy. *J Am Coll Cardiol.* 2005; 46: 1411-6.
25 Brown MS, Kovanen PT, Goldstein JL, Eeckels R, Vandenberghe K, van den Berghe H, et al. Prenatal diagnosis of homozygous familial hypercholesterolaemia. Expression of a genetic receptor disease in utero. *Lancet.* 1978; 1: 526-9.
26 Lieb W, Enserro DM, Larson MG, Vasan RS. Residual cardiovascular risk in individuals on lipid-lowering treatment: quantifying absolute and relative risk in the community. *Open Heart.* 2018; 5: e000722.
27 Fernandez-Friera L, Fuster V, Lopez-Melgar B, Oliva B, Garcia-Ruiz JM, Mendiguren J, et al. Normal LDL-Cholesterol Levels Are Associated With Subclinical Atherosclerosis in the Absence of Risk Factors. *J Am Coll Cardiol.* 2017; 70: 2979-91.
28 Ference BA, Graham I, Tokgozoglu L, Catapano AL. Reprint of: Impact of Lipids on Cardiovascular Health: JACC Health Promotion Series. *J Am Coll Cardiol.* 2018; 72: 2980-95.
29 Cooney M, Cooney MT, Maher V, Khan B, Leong T, Graham I. Improvement in the estimation of cardiovascular risk by carotid intima-medial thickness: A report from the Dublin Cardiohealth station study. *Prev Med Rep.* 2015; 2: 725-9.
30 Laufs U, Weingartner O. Pathological phenotypes of LDL particles. *Eur Heart J.* 2018; 39: 2574-6.
31 Laaksonen R, Ekroos K, Sysi-Aho M, Hilvo M, Vihervaara T, Kauhanen D, et al. Plasma ceramides predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes beyond LDL-cholesterol. *Eur Heart J.* 2016; 37: 1967-76.

32 Lemaitre RN, Jensen PN, Hoofnagle A, McKnight B, Fretts AM, King IB, et al. Plasma Ceramides and Sphingomyelins in Relation to Heart Failure Risk. *Circ Heart Fail.* 2019; 12: e005708.
33 Meeusen JW, Donato LJ, Bryant SC, Baudhuin LM, Berger PB, Jaffe AS. Plasma Ceramides. *Arterioscler Thromb Vasc Biol.* 2018; 38: 1933-9.
34 Hannun YA, Obeid LM. Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Biol.* 2018; 19: 175-91.
35 Borodzicz S, Czarzasta K, Kuch M, Cudnoch-Jedrzejewska A. Sphingolipids in cardiovascular diseases and metabolic disorders. *Lipids Health Dis.* 2015; 14: 55.
36 Schissel SL, Tweedie-Hardman J, Rapp JH, Graham G, Williams KJ, Tabas I. Rabbit aorta and human atherosclerotic lesions hydrolyze the sphingomyelin of retained low-density lipoprotein. Proposed role for arterial-wall sphingomyelinase in subendothelial retention and aggregation of atherogenic lipoproteins. *J Clin Invest.* 1996; 98: 1455-64.
37 Havulinna AS, Sysi-Aho M, Hilvo M, Kauhanen D, Hurme R, Ekroos K, et al. Circulating Ceramides Predict Cardiovascular Outcomes in the Population-Based FINRISK 2002 Cohort. *Arterioscler Thromb Vasc Biol.* 2016; 36: 2424-30.
38 Cheng JM, Suoniemi M, Kardys I, Vihervaara T, de Boer SP, Akkerhuis KM, et al. Plasma concentrations of molecular lipid species in relation to coronary plaque characteristics and cardiovascular outcome: Results of the ATHEROREMO-IVUS study. *Atherosclerosis.* 2015; 243: 560-6.
39 Hilvo M, Meikle PJ, Pedersen ER, Tell GS, Dhar I, Brenner H, et al. Development and validation of a ceramide- and phospholipid-based cardiovascular risk estimation score for coronary artery disease patients. *Eur Heart J.* 2019.
40 Volgman AS, Palaniappan LS, Aggarwal NT, Gupta M, Khandelwal A, Krishnan AV, et al. Atherosclerotic Cardiovascular Disease in South Asians in the United States: Epidemiology, Risk Factors, and Treatments: A Scientific Statement From the American Heart Association. *Circulation.* 2018; 138: e1-e34.
41 Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet.* 2004; 364: 937-52.
42 Forouhi NG, Sattar N, Tillin T, McKeigue PM, Chaturvedi N. Do known risk factors explain the higher coronary heart disease mortality in South Asian compared with European men? Prospective follow-up of the Southall and Brent studies, UK. *Diabetologia.* 2006; 49: 2580-8.
43 Anand SS, Yusuf S, Vuksan V, Devanesen S, Teo KK, Montague PA, et al. Differences in risk factors, atherosclerosis, and cardiovascular disease between ethnic groups in Canada: the Study of Health Assessment and Risk in Ethnic groups (SHARE). *Lancet.* 2000; 356: 279-84.
44 McKeigue PM, Ferrie JE, Pierpoint T, Marmot MG. Association of early-onset coronary heart disease in South Asian men with glucose intolerance and hyperinsulinemia. *Circulation.* 1993; 87: 152-61.
45 Rao AS, Knowles JW. Polygenic risk scores in coronary artery disease. *Curr Opin Cardiol.* 2019; 34: 435-40.

46 Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet.* 1994; 344: 1383-9.
47 Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med.* 1995; 333: 1301-7.
48 Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med.* 1996; 335: 1001-9.
49 Lim SY. Role of statins in coronary artery disease. *Chonnam Med J.* 2013; 49: 1-6.
50 Byrne P, Cullinan J, Smith SM. Statins for primary prevention of cardiovascular disease. *BMJ.* 2019; 367: l5674.
51 Taylor F, Huffman MD, Macedo AF, Moore TH, Burke M, Davey Smith G, et al. Statins for the primary prevention of cardiovascular disease. *Cochrane Database Syst Rev.* 2013 CD004816.
52 Pandor A, Ara RM, Tumur I, Wilkinson AJ, Paisley S, Duenas A, et al. Ezetimibe monotherapy for cholesterol lowering in 2,722 people: systematic review and meta-analysis of randomized controlled trials. *J Intern Med.* 2009; 265: 568-80.
53 Kastelein JJ, Akdim F, Stroes ES, Zwinderman AH, Bots ML, Stalenhoef AF, et al. Simvastatin with or without ezetimibe in familial hypercholesterolemia. *N Engl J Med.* 2008; 358: 1431-43.
54 Phan BA, Dayspring TD, Toth PP. Ezetimibe therapy: mechanism of action and clinical update. *Vasc Health Risk Manag.* 2012; 8: 415-27.
55 Sabatine MS, Giugliano RP, Keech AC, Honarpour N, Wiviott SD, Murphy SA, et al. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. *N Engl J Med.* 2017; 376: 1713-22.
56 Abifadel M, Varret M, Rabes JP, Allard D, Ouguerram K, Devillers M, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet.* 2003; 34: 154-6.
57 Schwartz GG, Steg PG, Szarek M, Bhatt DL, Bittner VA, Diaz R, et al. Alirocumab and Cardiovascular Outcomes after Acute Coronary Syndrome. *N Engl J Med.* 2018; 379: 2097-107.
58 Cholesterol Treatment Trialists C. Efficacy and safety of statin therapy in older people: a meta-analysis of individual participant data from 28 randomised controlled trials. *Lancet.* 2019; 393: 407-15.
59 Weng TC, Yang YH, Lin SJ, Tai SH. A systematic review and meta-analysis on the therapeutic equivalence of statins. *J Clin Pharm Ther.* 2010; 35: 139-51.
60 Blom DJ, Hala T, Bolognese M, Lillestol MJ, Toth PD, Burgess L, et al. A 52-week placebo-controlled trial of evolocumab in hyperlipidemia. *N Engl J Med.* 2014; 370: 1809-19.
61 Koren MJ, Sabatine MS, Giugliano RP, Langslet G, Wiviott SD, Ruzza A, et al. Long-Term Efficacy and Safety of Evolocumab in Patients With Hypercholesterolemia. *J Am Coll Cardiol.* 2019; 74: 2132-46.

62 Cannon CP, Blazing MA, Giugliano RP, McCagg A, White JA, Theroux P, et al. Ezetimibe Added to Statin Therapy after Acute Coronary Syndromes. *N Engl J Med.* 2015; 372: 2387-97.
63 Windaus A. Über den Gehalt normaler und atheromatöser Aorten an Cholesterin und Cholesterinestern. In english: About the content of normal and atheromatous aorta of cholesterol and cholesterol esters. *Hoppe-Seyler´s Zeitschrift für physiologische Chemie.* 1910; 67: 174-6.
64 Anitschkow N. CS. Classics in arteriosclerosis research: On experimental cholesterin steatosis and its significance in the origin of some pathological processes by N. Anitschkow and S. Chalatow, translated by Mary Z. Pelias, 1913. *Arteriosclerosis.* 1983; 3: 178-82.
65 Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol.* 1995; 15: 551-61.
66 Boren J, Williams KJ. The central role of arterial retention of cholesterol-rich apolipoprotein-B-containing lipoproteins in the pathogenesis of atherosclerosis: a triumph of simplicity. *Curr Opin Lipidol.* 2016; 27: 473-83.
67 Brown RA, Shantsila E, Varma C, Lip GY. Current Understanding of Atherogenesis. *Am J Med.* 2017; 130: 268-82.
68 Ross R, Glomset J, Harker L. Response to injury and atherogenesis. *Am J Pathol.* 1977; 86: 675-84.
69 Sloop CH, Dory L, Roheim PS. Interstitial fluid lipoproteins. *J Lipid Res.* 1987; 28: 225-37.
70 Davies PF. Flow-mediated endothelial mechanotransduction. *Physiol Rev.* 1995; 75: 519-60.
71 Gimbrone MA, Jr., Topper JN, Nagel T, Anderson KR, Garcia-Cardena G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci.* 2000; 902: 230-9; discussion 9-40.
72 Williams KJ, Tabas I. Lipoprotein retention - and clues for atheroma regression. *Arterioscler Thromb Vasc Biol.* 2005; 25: 1536-40.
73 Skalen K, Gustafsson M, Rydberg EK, Hulten LM, Wiklund O, Innerarity TL, et al. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature.* 2002; 417: 750-4.
74 Hurt-Camejo E, Camejo G. ApoB-100 Lipoprotein Complex Formation with Intima Proteoglycans as a Cause of Atherosclerosis and Its Possible Ex Vivo Evaluation as a Disease Biomarker. *J Cardiovasc Dev Dis.* 2018; 5.
75 Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature.* 2011; 473: 317-25.
76 Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nat Immunol.* 2011; 12: 204-12.
77 Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation.* 2007; 116: 1832-44.
78 Iverius PH. The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans. *J Biol Chem.* 1972; 247: 2607-13.

79 Tsai NW, Lee LH, Huang CR, Chang WN, Chang YT, Su YJ, et al. Statin therapy reduces oxidized low density lipoprotein level, a risk factor for stroke outcome. *Crit Care.* 2014; 18: R16.
80 Pirillo A, Norata GD, Catapano AL. LOX-1, OxLDL, and atherosclerosis. *Mediators Inflamm.* 2013; 2013: 152786.
81 Trpkovic A, Resanovic I, Stanimirovic J, Radak D, Mousa SA, Cenic-Milosevic D, et al. Oxidized low-density lipoprotein as a biomarker of cardiovascular diseases. *Crit Rev Clin Lab Sci.* 2015; 52: 70-85.
82 Öörni K, Pentikäinen MO, Ala-Korpela M, Kovanen PT. Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions. *J Lipid Res.* 2000; 41: 1703-14.
83 Sneck M, Nguyen SD, Pihlajamaa T, Yohannes G, Riekkola ML, Milne R, et al. Conformational changes of apoB-100 in SMase-modified LDL mediate formation of large aggregates at acidic pH. *J Lipid Res.* 2012; 53: 1832-9.
84 Schissel SL, Jiang X, Tweedie-Hardman J, Jeong T, Camejo EH, Najib J, et al. Secretory sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. *J Biol Chem.* 1998; 273: 2738-46.
85 Devlin CM, Leventhal AR, Kuriakose G, Schuchman EH, Williams KJ, Tabas I. Acid sphingomyelinase promotes lipoprotein retention within early atheromata and accelerates lesion progression. *Arterioscler. Thromb. Vasc. Biol.* 2008; 28: 1723-30.
86 Hoff HF, Morton RE. Lipoproteins containing apo B extracted from human aortas. Structure and function. *Ann N Y Acad Sci.* 1985; 454: 183-94.
87 Lehti S, Nguyen D, Belevich I, Vihinen H, Soliymani R, Käkelä R, et al. Extracellular lipid accumulates in human carotid arteries as distinct three-dimensional structures with proinflammatory properties. *Am J Pathol.* 2018; 188: 525-38.
88 Oorni K, Posio P, Ala-Korpela M, Jauhiainen M, Kovanen PT. Sphingomyelinase induces aggregation and fusion of small very low-density lipoprotein and intermediate-density lipoprotein particles and increases their retention to human arterial proteoglycans. *Arterioscler Thromb Vasc Biol.* 2005; 25: 1678-83.
89 Tabas I, Li Y, Brocia RW, Xu SW, Swenson TL, Williams KJ. Lipoprotein lipase and sphingomyelinase synergistically enhance the association of atherogenic lipoproteins with smooth muscle cells and extracellular matrix. A possible mechanism for low density lipoprotein and lipoprotein(a) retention and macrophage foam cell formation. *J Biol Chem.* 1993; 268: 20419-32.
90 Suits AG, Chait A, Aviram M, Heinecke JW. Phagocytosis of aggregated lipoprotein by macrophages: low density lipoprotein receptor-dependent foam-cell formation. *Proc Natl Acad Sci U S A.* 1989; 86: 2713-7.
91 Haka AS, Grosheva I, Chiang E, Buxbaum AR, Baird BA, Pierini LM, et al. Macrophages create an acidic extracellular hydrolytic compartment to digest aggregated lipoproteins. *Mol Biol Cell.* 2009; 20: 4932-40.
92 Singh RK, Haka AS, Bhardwaj P, Zha X, Maxfield FR. Dynamic Actin Reorganization and Vav/Cdc42-Dependent Actin Polymerization Promote Macrophage Aggregated LDL (Low-Density Lipoprotein) Uptake and Catabolism. *Arterioscler Thromb Vasc Biol.* 2019; 39: 137-49.

93 Rajamaki K, Lappalainen J, Oorni K, Valimaki E, Matikainen S, Kovanen PT, et al. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS One.* 2010; 5: e11765.
94 Suriyaphol P, Fenske D, Zahringer U, Han SR, Bhakdi S, Husmann M. Enzymatically modified nonoxidized low-density lipoprotein induces interleukin-8 in human endothelial cells: role of free fatty acids. *Circulation.* 2002; 106: 2581-7.
95 Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, Jr., et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature.* 1999; 398: 718-23.
96 Pentikainen MO, Oorni K, Ala-Korpela M, Kovanen PT. Modified LDL - trigger of atherosclerosis and inflammation in the arterial intima. *J Intern Med.* 2000; 247: 359-70.
97 Ketelhuth DFJ, Hansson GK. Adaptive Response of T and B Cells in Atherosclerosis. *Circ Res.* 2016; 118: 668-78.
98 Ketelhuth DF, Back M. The role of matrix metalloproteinases in atherothrombosis. *Curr Atheroscler Rep.* 2011; 13: 162-9.
99 Hansson GK, Libby P, Tabas I. Inflammation and plaque vulnerability. *J Intern Med.* 2015; 278: 483-93.
100 Back M, Yurdagul A, Jr., Tabas I, Oorni K, Kovanen PT. Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities. *Nat Rev Cardiol.* 2019; 16: 389-406.
101 Otsuka F, Yasuda S, Noguchi T, Ishibashi-Ueda H. Pathology of coronary atherosclerosis and thrombosis. *Cardiovasc Diagn Ther.* 2016; 6: 396-408.
102 Tabas I, Lichtman AH. Monocyte-Macrophages and T Cells in Atherosclerosis. *Immunity.* 2017; 47: 621-34.
103 Witztum JL. You are right too! *J Clin Invest.* 2005; 115: 2072-5.
104 Kannel WB, Castelli WP, Gordon T, McNamara PM. Serum cholesterol, lipoproteins, and the risk of coronary heart disease. The Framingham study. *Ann Intern Med.* 1971; 74: 1-12.
105 Kovanen PP, M.; Viikari, J. *Dyslipidemiat*. (Duodecim, 2009).
106 Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT, Ala-Korpela M. Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. *Biochim Biophys Acta.* 2000; 1488: 189-210.
107 Wolska A, Dunbar RL, Freeman LA, Ueda M, Amar MJ, Sviridov DO, et al. Apolipoprotein C-II: New findings related to genetics, biochemistry, and role in triglyceride metabolism. *Atherosclerosis.* 2017; 267: 49-60.
108 Olivecrona G. Role of lipoprotein lipase in lipid metabolism. *Curr Opin Lipidol.* 2016; 27: 233-41.
109 Mahley RW. Apolipoprotein E: from cardiovascular disease to neurodegenerative disorders. *J Mol Med (Berl).* 2016; 94: 739-46.
110 Ferrebee CB, Dawson PA. Metabolic effects of intestinal absorption and enterohepatic cycling of bile acids. *Acta Pharm Sin B.* 2015; 5: 129-34.
111 Sehayek E. Genetic regulation of cholesterol absorption and plasma plant sterol levels: commonalities and differences. *J Lipid Res.* 2003; 44: 2030-8.

112 Hofmann AF. The continuing importance of bile acids in liver and intestinal disease. *Arch Intern Med.* 1999; 159: 2647-58.
113 Mabuchi H, Nohara A, Inazu A. Cholesteryl ester transfer protein (CETP) deficiency and CETP inhibitors. *Mol Cells.* 2014; 37: 777-84.
114 Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ Res.* 2005; 96: 1221-32.
115 Feingold KR, Grunfeld C. in *Endotext* (eds K. R. Feingold *et al.*) (2000).
116 Huang LH, Elvington A, Randolph GJ. The role of the lymphatic system in cholesterol transport. *Front Pharmacol.* 2015; 6: 182.
117 Marcucci R, Liotta AA, Cellai AP, Rogolino A, Gori AM, Giusti B, et al. Increased plasma levels of lipoprotein(a) and the risk of idiopathic and recurrent venous thromboembolism. *Am J Med.* 2003; 115: 601-5.
118 Boffa MB, Koschinsky ML. Lipoprotein (a): truly a direct prothrombotic factor in cardiovascular disease? *J Lipid Res.* 2016; 57: 745-57.
119 Boffa MB, Marar TT, Yeang C, Viney NJ, Xia S, Witztum JL, et al. Potent reduction of plasma lipoprotein(a) with an antisense oligonucleotide in human subjects does not affect ex vivo fibrinolysis. *J Lipid Res.* 2019.
120 Suwa S, Ogita M, Miyauchi K, Sonoda T, Konishi H, Tsuboi S, et al. Impact of Lipoprotein (a) on Long-Term Outcomes in Patients with Coronary Artery Disease Treated with Statin After a First Percutaneous Coronary Intervention. *J Atheroscler Thromb.* 2017; 24: 1125-31.
121 Keys A, Menotti A, Aravanis C, Blackburn H, Djordevic BS, Buzina R, et al. The seven countries study: 2,289 deaths in 15 years. *Prev Med.* 1984; 13: 141-54.
122 Wilson JD, Lindsey CA, Dietschy JM. Influence of dietary cholesterol on cholesterol metabolism. *Ann N Y Acad Sci.* 1968; 149: 808-21.
123 Piepoli MF, Hoes AW, Agewall S, Albus C, Brotons C, Catapano AL, et al. 2016 European Guidelines on cardiovascular disease prevention in clinical practice: The Sixth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of 10 societies and by invited experts)Developed with the special contribution of the European Association for Cardiovascular Prevention & Rehabilitation (EACPR). *Eur Heart J.* 2016; 37: 2315-81.
124 Sacks FM, Lichtenstein AH, Wu JHY, Appel LJ, Creager MA, Kris-Etherton PM, et al. Dietary Fats and Cardiovascular Disease: A Presidential Advisory From the American Heart Association. *Circulation.* 2017; 136: e1-e23.
125 Forouhi NG, Unwin N. Global diet and health: old questions, fresh evidence, and new horizons. *Lancet.* 2019; 393: 1916-8.
126 Gambardella J, Santulli G. Integrating diet and inflammation to calculate cardiovascular risk. *Atherosclerosis.* 2016; 253: 258-61.
127 Mozaffarian D, Appel LJ, Van Horn L. Components of a cardioprotective diet: new insights. *Circulation.* 2011; 123: 2870-91.
128 Mensink RP. Effects of saturated fatty acids on serum lipids and lipoproteins: a systematic review and regression analysis. *Geneva: World Health Organization.* 2016.

129 Forouhi NG, Krauss RM, Taubes G, Willett W. Dietary fat and cardiometabolic health: evidence, controversies, and consensus for guidance. *BMJ.* 2018; 361: k2139.
130 Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. *N Engl J Med.* 2018; 378: e34.
131 Dehghan M, Mente A, Zhang X, Swaminathan S, Li W, Mohan V, et al. Associations of fats and carbohydrate intake with cardiovascular disease and mortality in 18 countries from five continents (PURE): a prospective cohort study. *Lancet.* 2017; 390: 2050-62.
132 Gianos E, Williams KA, Freeman AM, Kris-Etherton P, Aggarwal M. How Pure is PURE? Dietary Lessons Learned and Not Learned From the PURE Trials. *Am J Med.* 2018; 131: 457-8.
133 Malhotra A, Sahdev N, Sharma S. Diet and Nutrition after the PURE study. *Eur Heart J.* 2018; 39: 1503-4.
134 Schaefer EJ, Augustin JL, Schaefer MM, Rasmussen H, Ordovas JM, Dallal GE, et al. Lack of efficacy of a food-frequency questionnaire in assessing dietary macronutrient intakes in subjects consuming diets of known composition. *Am J Clin Nutr.* 2000; 71: 746-51.
135 Pan M, Cederbaum AI, Zhang YL, Ginsberg HN, Williams KJ, Fisher EA. Lipid peroxidation and oxidant stress regulate hepatic apolipoprotein B degradation and VLDL production. *J Clin Invest.* 2004; 113: 1277-87.
136 McNamara DJ, Kolb R, Parker TS, Batwin H, Samuel P, Brown CD, et al. Heterogeneity of cholesterol homeostasis in man. Response to changes in dietary fat quality and cholesterol quantity. *J Clin Invest.* 1987; 79: 1729-39.
137 Manninen S, Lankinen M, de Mello V, Agren J, Laaksonen D, Schwab U, et al. The effect of camelina sativa oil and fish intakes on fatty acid compositions of blood lipid fractions. *Nutr Metab Cardiovasc Dis.* 2019; 29: 51-61.
138 Nguyen TT. The cholesterol-lowering action of plant stanol esters. *J Nutr.* 1999; 129: 2109-12.
139 Rosin S, Ojansivu I, Kopu A, Keto-Tokoi M, Gylling H. Optimal Use of Plant Stanol Ester in the Management of Hypercholesterolemia. *Cholesterol.* 2015; 2015: 706970.
140 Athyros VG, Kakafika AI, Papageorgiou AA, Tziomalos K, Peletidou A, Vosikis C, et al. Effect of a plant stanol ester-containing spread, placebo spread, or Mediterranean diet on estimated cardiovascular risk and lipid, inflammatory and haemostatic factors. *Nutr Metab Cardiovasc Dis.* 2011; 21: 213-21.
141 Valsta LM, Lemstrom A, Ovaskainen ML, Lampi AM, Toivo J, Korhonen T, et al. Estimation of plant sterol and cholesterol intake in Finland: quality of new values and their effect on intake. *Br J Nutr.* 2004; 92: 671-8.
142 Ostlund RE, Jr., McGill JB, Zeng CM, Covey DF, Stearns J, Stenson WF, et al. Gastrointestinal absorption and plasma kinetics of soy Delta(5)-phytosterols and phytostanols in humans. *Am J Physiol Endocrinol Metab.* 2002; 282: E911-6.
143 Stanhope KL, Medici V, Bremer AA, Lee V, Lam HD, Nunez MV, et al. A dose-response study of consuming high-fructose corn syrup-sweetened beverages on

lipid/lipoprotein risk factors for cardiovascular disease in young adults. *Am J Clin Nutr.* 2015; 101: 1144-54.

144 Stanhope KL, Schwarz JM, Keim NL, Griffen SC, Bremer AA, Graham JL, et al. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *J Clin Invest.* 2009; 119: 1322-34.

145 Welsh JA, Sharma A, Cunningham SA, Vos MB. Consumption of added sugars and indicators of cardiovascular disease risk among US adolescents. *Circulation.* 2011; 123: 249-57.

146 Fung TT, Malik V, Rexrode KM, Manson JE, Willett WC, Hu FB. Sweetened beverage consumption and risk of coronary heart disease in women. *Am J Clin Nutr.* 2009; 89: 1037-42.

147 Retterstol K, Svendsen M, Narverud I, Holven KB. Effect of low carbohydrate high fat diet on LDL cholesterol and gene expression in normal-weight, young adults: A randomized controlled study. *Atherosclerosis.* 2018; 279: 52-61.

148 Fung TT, van Dam RM, Hankinson SE, Stampfer M, Willett WC, Hu FB. Low-carbohydrate diets and all-cause and cause-specific mortality: two cohort studies. *Ann Intern Med.* 2010; 153: 289-98.

149 Lagiou P, Sandin S, Lof M, Trichopoulos D, Adami HO, Weiderpass E. Low carbohydrate-high protein diet and incidence of cardiovascular diseases in Swedish women: prospective cohort study. *BMJ.* 2012; 344: e4026.

150 Sacks FM, Svetkey LP, Vollmer WM, Appel LJ, Bray GA, Harsha D, et al. Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group. *N Engl J Med.* 2001; 344: 3-10.

151 He FJ, MacGregor GA. Effect of modest salt reduction on blood pressure: a meta-analysis of randomized trials. Implications for public health. *J Hum Hypertens.* 2002; 16: 761-70.

152 Aburto NJ, Hanson S, Gutierrez H, Hooper L, Elliott P, Cappuccio FP. Effect of increased potassium intake on cardiovascular risk factors and disease: systematic review and meta-analyses. *BMJ.* 2013; 346: f1378.

153 Manson JE, Cook NR, Lee IM, Christen W, Bassuk SS, Mora S, et al. Marine n-3 Fatty Acids and Prevention of Cardiovascular Disease and Cancer. *N Engl J Med.* 2019; 380: 23-32.

154 Alpha-Tocopherol BCCPSG. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med.* 1994; 330: 1029-35.

155 Threapleton DE, Greenwood DC, Evans CE, Cleghorn CL, Nykjaer C, Woodhead C, et al. Dietary fibre intake and risk of cardiovascular disease: systematic review and meta-analysis. *BMJ.* 2013; 347: f6879.

156 Zampelas A, Magriplis E. Dietary patterns and risk of cardiovascular diseases: a review of the evidence. *Proc Nutr Soc.* 2019 1-8.

157 Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, et al. Primary prevention of cardiovascular disease with a Mediterranean diet. *N Engl J Med.* 2013; 368: 1279-90.

158 Stefler D, Malyutina S, Kubinova R, Pajak A, Peasey A, Pikhart H, et al. Mediterranean diet score and total and cardiovascular mortality in Eastern Europe: the HAPIEE study. *Eur J Nutr.* 2017; 56: 421-9.
159 Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, et al. Retraction and Republication: Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. N Engl J Med 2013;368:1279-90. *N Engl J Med.* 2018; 378: 2441-2.
160 Becker W, Lyhne N, Pedersen AN, Aro A, Fogelholm M, Phorsdottir I, et al. Nordic Nutrition Recommendations 2004 - integrating nutrition and physical activity. *Scandinavian Journal of Nutrition.* 2004; 48: 178-87.
161 Daneshzad E, Emami S, Darooghegi Mofrad M, Saraf-Bank S, Surkan PJ, Azadbakht L. Association of modified Nordic diet with cardiovascular risk factors among type 2 diabetes patients: a cross-sectional study. *J Cardiovasc Thorac Res.* 2018; 10: 153-61.
162 Hansen CP, Overvad K, Kyro C, Olsen A, Tjonneland A, Johnsen SP, et al. Adherence to a Healthy Nordic Diet and Risk of Stroke: A Danish Cohort Study. *Stroke.* 2017; 48: 259-64.
163 Key TJ, Appleby PN, Bradbury KE, Sweeting M, Wood A, Johansson I, et al. Consumption of Meat, Fish, Dairy Products, and Eggs and Risk of Ischemic Heart Disease. *Circulation.* 2019; 139: 2835-45.
164 Song M, Fung TT, Hu FB, Willett WC, Longo VD, Chan AT, et al. Association of Animal and Plant Protein Intake With All-Cause and Cause-Specific Mortality. *JAMA Intern Med.* 2016; 176: 1453-63.
165 Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med.* 2013; 19: 576-85.
166 Aromaa A, Koskinen, S., (eds). . Health and Functional capasity in Finland. Baseline results of the Health 2000 Health Examination Survey. *Publication of the National Public Health Institute.* 2004; B12: http://www.terveys2000.fi/julkaisut/baseline.pdf.
167 Uusitupa M, Hermansen K, Savolainen MJ, Schwab U, Kolehmainen M, Brader L, et al. Effects of an isocaloric healthy Nordic diet on insulin sensitivity, lipid profile and inflammation markers in metabolic syndrome -- a randomized study (SYSDIET). *J Intern Med.* 2013; 274: 52-66.
168 Vaara S, Nieminen MS, Lokki ML, Perola M, Pussinen PJ, Allonen J, et al. Cohort Profile: the Corogene study. *Int J Epidemiol.* 2012; 41: 1265-71.
169 Baruch A, Mosesova S, Davis JD, Budha N, Vilimovskij A, Kahn R, et al. Effects of RG7652, a Monoclonal Antibody Against PCSK9, on LDL-C, LDL-C Subfractions, and Inflammatory Biomarkers in Patients at High Risk of or With Established Coronary Heart Disease (from the Phase 2 EQUATOR Study). *Am J Cardiol.* 2017; 119: 1576-83.
170 Luukkonen PK, Sadevirta S, Zhou Y, Kayser B, Ali A, Ahonen L, et al. Saturated Fat Is More Metabolically Harmful for the Human Liver Than Unsaturated Fat or Simple Sugars. *Diabetes Care.* 2018; 41: 1732-9.
171 Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International

Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation.* 2009; 120: 1640-5.

172 World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA.* 2013; 310: 2191-4.

173 Chapman MJ, Goldstein S, Lagrange D, Laplaud PM. A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J Lipid Res.* 1981; 22: 339-58.

174 Hallberg C, Haden M, Bergstrom M, Hanson G, Pettersson K, Westerlund C, et al. Lipoprotein fractionation in deuterium oxide gradients: a procedure for evaluation of antioxidant binding and susceptibility to oxidation. *J Lipid Res.* 1994; 35: 1-9.

175 Sakurai T, Trirongjitmoah S, Nishibata Y, Namita T, Tsuji M, Hui SP, et al. Measurement of lipoprotein particle sizes using dynamic light scattering. *Ann Clin Biochem.* 2010; 47: 476-81.

176 Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957; 226: 497-509.

177 Duffin KL, Henion JD, Shieh JJ. Electrospray and tandem mass spectrometric characterization of acylglycerol mixtures that are dissolved in nonpolar solvents. *Anal Chem.* 1991; 63: 1781-8.

178 Hsu FF, Turk J. Structural determination of glycosphingolipids as lithiated adducts by electrospray ionization mass spectrometry using low-energy collisional-activated dissociation on a triple stage quadrupole instrument. *J Am Soc Mass Spectrom.* 2001; 12: 61-79.

179 Murphy RC, Axelsen PH. Mass spectrometric analysis of long-chain lipids. *Mass Spectrom Rev.* 2011; 30: 579-99.

180 Kauhanen D, Sysi-Aho M, Koistinen KM, Laaksonen R, Sinisalo J, Ekroos K. Development and validation of a high-throughput LC-MS/MS assay for routine measurement of molecular ceramides. *Anal Bioanal Chem.* 2016; 408: 3475-83.

181 Hurt E, Bondjers G, Camejo G. Interaction of LDL with human arterial proteoglycans stimulates its uptake by human monocyte-derived macrophages. *J Lipid Res.* 1990; 31: 443-54.

182 Oorni K, Pentikainen MO, Annila A, Kovanen PT. Oxidation of low density lipoprotein particles decreases their ability to bind to human aortic proteoglycans. Dependence on oxidative modification of the lysine residues. *J Biol Chem.* 1997; 272: 21303-11.

183 Nguyen SD, Oorni K, Lee-Rueckert M, Pihlajamaa T, Metso J, Jauhiainen M, et al. Spontaneous remodeling of HDL particles at acidic pH enhances their capacity to induce cholesterol efflux from human macrophage foam cells. *J Lipid Res.* 2012; 53: 2115-25.

184 Nakanishi S, Vikstedt R, Soderlund S, Lee-Rueckert M, Hiukka A, Ehnholm C, et al. Serum, but not monocyte macrophage foam cells derived from low HDL-C subjects, displays reduced cholesterol efflux capacity. *J Lipid Res.* 2009; 50: 183-92.

185 Plihtari R, Kovanen PT, Oorni K. Acidity increases the uptake of native LDL by human monocyte-derived macrophages. *Atherosclerosis.* 2011; 217: 401-6.

186 Hermansson A, Ketelhuth DF, Strodthoff D, Wurm M, Hansson EM, Nicoletti A, et al. Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis. *J Exp Med.* 2010; 207: 1081-93.
187 Melchior JT, Sawyer JK, Kelley KL, Shah R, Wilson MD, Hantgan RR, et al. LDL particle core enrichment in cholesteryl oleate increases proteoglycan binding and promotes atherosclerosis. *J Lipid Res.* 2013; 54: 2495-503.
188 Haimi P, Uphoff A, Hermansson M, Somerharju P. Software tools for analysis of mass spectrometric lipidome data. *Anal Chem.* 2006; 78: 8324-31.
189 Folch J, Lees M, Stanley GHS. A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. 1957; 226: 497-509.
190 Tanaka M, Dhanasekaran P, Nguyen D, Ohta S, Lund-Katz S, Phillips MC, et al. Contributions of the N- and C-terminal helical segments to the lipid-free structure and lipid interaction of apolipoprotein A-I. *Biochemistry.* 2006; 45: 10351-8.
191 Morrow JA, Segall ML, Lund-Katz S, Phillips MC, Knapp M, Rupp B, et al. Differences in stability among the human apolipoprotein E isoforms determined by the amino-terminal domain. *Biochemistry.* 2000; 39: 11657-66.
192 Borén J, Williams KJ. The central role of arterial retention of cholesterol-rich apolipoprotein-B-containing lipoproteins in the pathogenesis of atherosclerosis: a triumph of simplicity. *Curr Opin Lipidol.* 2016; 27: 473-83.
193 Khokha R, Murthy A, Weiss A. Metalloproteinases and their natural inhibitors in inflammation and immunity. *Nat Rev Immunol.* 2013; 13: 649-65.
194 Bartlett B, Ludewick HP, Misra A, Lee S, Dwivedi G. Macrophages and T cells in atherosclerosis: a translational perspective. *Am J Physiol Heart Circ Physiol.* 2019; 317: H375-H86.
195 Rodrigueza WV, Mazany KD, Essenburg AD, Pape ME, Rea TJ, Bisgaier CL, et al. Large versus small unilamellar vesicles mediate reverse cholesterol transport in vivo into two distinct hepatic metabolic pools. Implications for the treatment of atherosclerosis. *Arterioscler Thromb Vasc Biol.* 1997; 17: 2132-9.
196 Hojjati MR, Li Z, Zhou H, Tang S, Huan C, Ooi E, et al. Effect of myriocin on plasma sphingolipid metabolism and atherosclerosis in apoE-deficient mice. *J Biol Chem.* 2005; 280: 10284-9.
197 Willner EL, Tow B, Buhman KK, Wilson M, Sanan DA, Rudel LL, et al. Deficiency of acyl CoA:cholesterol acyltransferase 2 prevents atherosclerosis in apolipoprotein E-deficient mice. *Proc Natl Acad Sci U S A.* 2003; 100: 1262-7.
198 Cases S, Novak S, Zheng YW, Myers HM, Lear SR, Sande E, et al. ACAT-2, a second mammalian acyl-CoA:cholesterol acyltransferase. Its cloning, expression, and characterization. *J Biol Chem.* 1998; 273: 26755-64.
199 Lee RG, Willingham MC, Davis MA, Skinner KA, Rudel LL. Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. *J Lipid Res.* 2000; 41: 1991-2001.
200 Ohshiro T, Matsuda D, Sakai K, Degirolamo C, Yagyu H, Rudel LL, et al. Pyripyropene A, an acyl-coenzyme A:cholesterol acyltransferase 2-selective inhibitor, attenuates hypercholesterolemia and atherosclerosis in murine models of hyperlipidemia. *Arterioscler Thromb Vasc Biol.* 2011; 31: 1108-15.

201 Lu M, Gursky O. Aggregation and fusion of low-density lipoproteins in vivo and in vitro. *Biomol Concepts.* 2013; 4: 501-18.
202 Khot UN, Khot MB, Bajzer CT, Sapp SK, Ohman EM, Brener SJ, et al. Prevalence of conventional risk factors in patients with coronary heart disease. *JAMA.* 2003; 290: 898-904.
203 Myerburg RJ. Sudden cardiac death: exploring the limits of our knowledge. *J Cardiovasc Electrophysiol.* 2001; 12: 369-81.
204 Myerburg RJ, Junttila MJ. Sudden cardiac death caused by coronary heart disease. *Circulation.* 2012; 125: 1043-52.
205 Duncan JS, Duncan EK, Schofield G. Accuracy of body mass index (BMI) thresholds for predicting excess body fat in girls from five ethnicities. *Asia Pac J Clin Nutr.* 2009; 18: 404-11.
206 Deurenberg-Yap M, Schmidt G, van Staveren WA, Deurenberg P. The paradox of low body mass index and high body fat percentage among Chinese, Malays and Indians in Singapore. *Int J Obes Relat Metab Disord.* 2000; 24: 1011-7.
207 Magnusdottir OK, Landberg R, Gunnarsdottir I, Cloetens L, Akesson B, Onning G, et al. Plasma alkylresorcinols reflect important whole-grain components of a healthy Nordic diet. *J Nutr.* 2013; 143: 1383-90.
208 Hilvo M, Simolin H, Metso J, Ruuth M, Oorni K, Jauhiainen M, et al. PCSK9 inhibition alters the lipidome of plasma and lipoprotein fractions. *Atherosclerosis.* 2018; 269: 159-65.
209 Öörni K, Kovanen PT. PLA2-V: a real player in atherogenesis. *Arterioscler Thromb Vasc Biol.* 2007; 27: 445-7.
210 Heffron SP, Ruuth MK, Hernandez G, Rodriguez C, Oorni K, Berger JS. Abstract 12993: Low Density Lipoprotein Aggregation is Increased and Predictive of Major Adverse Cardiovascular Events in Patients With Peripheral Artery Disease Undergoing Lower Extremity Revascularization. *Circulation.* 2019; 140: A12993-A.
211 Merrill AH, Jr., Wang E, Mullins RE. Kinetics of long-chain (sphingoid) base biosynthesis in intact LM cells: effects of varying the extracellular concentrations of serine and fatty acid precursors of this pathway. *Biochemistry.* 1988; 27: 340-5.
212 Messmer TO, Wang E, Stevens VL, Merrill AH, Jr. Sphingolipid biosynthesis by rat liver cells: effects of serine, fatty acids and lipoproteins. *J Nutr.* 1989; 119: 534-8.
213 Manninen S, Lankinen M, Erkkila A, Nguyen SD, Ruuth M, de Mello V, et al. The effect of intakes of fish and Camelina sativa oil on atherogenic and anti-atherogenic functions of LDL and HDL particles: A randomized controlled trial. *Atherosclerosis.* 2019; 281: 56-61.
214 Jones PJ, MacKay DS, Senanayake VK, Pu S, Jenkins DJ, Connelly PW, et al. High-oleic canola oil consumption enriches LDL particle cholesteryl oleate content and reduces LDL proteoglycan binding in humans. *Atherosclerosis.* 2015; 238: 231-8.
215 Hannon BA, Thompson SV, An R, Teran-Garcia M. Clinical Outcomes of Dietary Replacement of Saturated Fatty Acids with Unsaturated Fat Sources in Adults with Overweight and Obesity: A Systematic Review and Meta-Analysis of Randomized Control Trials. *Ann Nutr Metab.* 2017; 71: 107-17.

216 Sundfor TM, Svendsen M, Heggen E, Dushanov S, Klemsdal TO, Tonstad S. BMI modifies the effect of dietary fat on atherogenic lipids: a randomized clinical trial. *Am J Clin Nutr.* 2019; 110: 832-41.
217 Lefevre M, Champagne CM, Tulley RT, Rood JC, Most MM. Individual variability in cardiovascular disease risk factor responses to low-fat and low-saturated-fat diets in men: body mass index, adiposity, and insulin resistance predict changes in LDL cholesterol. *Am J Clin Nutr.* 2005; 82: 957-63; quiz 1145-6.
218 Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. *J Clin Invest.* 2008; 118: 2992-3002.
219 Gylling H, Miettinen TA. Cholesterol absorption, synthesis, and LDL metabolism in NIDDM. *Diabetes Care.* 1997; 20: 90-5.
220 Miettinen TA, Gylling H. Cholesterol absorption efficiency and sterol metabolism in obesity. *Atherosclerosis.* 2000; 153: 241-8.
221 Wang J, Zhang L, Xiao R, Li Y, Liao S, Zhang Z, et al. Plasma lipidomic signatures of spontaneous obese rhesus monkeys. *Lipids Health Dis.* 2019; 18: 8.
222 Pietilainen KH, Rog T, Seppanen-Laakso T, Virtue S, Gopalacharyulu P, Tang J, et al. Association of lipidome remodeling in the adipocyte membrane with acquired obesity in humans. *PLoS Biol.* 2011; 9: e1000623.
223 Musa-Veloso K, Poon TH, Elliot JA, Chung C. A comparison of the LDL-cholesterol lowering efficacy of plant stanols and plant sterols over a continuous dose range: results of a meta-analysis of randomized, placebo-controlled trials. *Prostaglandins Leukot Essent Fatty Acids.* 2011; 85: 9-28.
224 Ruuth M, Luukkonen P, Sädevirta S, Kovanen P, Simonen P, Gylling H, et al. Dietary Saturated Fats Increase And Plant Stanol Esters Decreases Ldl Aggregation. *Atherosclerosis.* 2019; 287: e82.